# Original Article
# Involvement of macrophage migration inhibitory factor in the pathogenesis of idiopathic orbital inflammatory pseudotumor

Pengxiang Zhao[1*], Jianmin Ma[2*], Lei Shang[1], Adzavon Yao Mawulikplimi[1], Xiaoyue Wang[1], Yanan Wu[1], Fei Xie[1], Danying Chen[3], Linqi Yang[1], Qinjian Li[1], Xuemei Ma[1]

[1]College of Life Science and Bio-engineering, Beijing University of Technology, Beijing 100124, P. R. China; [2]Beijing Ophthalmology & Vision Science Key Lab, Beijing Tongren Eye center, Beijing Tongren Hospital, Capital Medical University, Beijing 100730, P. R. China; [3]Beijing Ditan Hospital, Capital Medical University, Beijing 100015, P. R. China. *Co-first authors.

**Abstract:** Increasing evidences suggested macrophage migration inhibitory factor (MIF) was important in biological activities of inflammatory disease, cancer genesis and the transition process from inflammation to tumor. In our study, we raised the missing link between MIF and pathogenesis of Idiopathic Orbital Inflammatory Pseudotumor (IOIP). IOIP samples were assigned for bio-plex measurement of 41 (human cytokines, chemokines and growth factors) and 17 cytokines (Th17 related cytokines) in plasma and tissue, respectively. MIF was the most elevated serological cytokine (IOIP = 30060±4785 pg/mL; Normal Donor = 1700±63 pg/mL). Microarray analysis for MIF receptor genes in tissue mRNA revealed that *CD74* and *CXCR4* were up-regulated comparing with *CD44* and *CXCR2*. Moreover, the expression level of MIF and its receptors (CD74, CXCR4) were also confirmed in tissue proteins by Western Blotting and immunofluorescence. We further found that the MIF downstream AKT signaling pathway was activated, targeting at phosphorylated-AKT, p53, bcl-2, p65, and p50 monomers. Analysis of the Single nucleotide polymorphism test revealed that MIF contributed at the genetic level, where MIF-173C and MIF-794 CATT[7] alleles were possibly dangerous factors, while MIF-794 CATT[6] allele may be a protective factor. This explained the high expression degree of MIF in affective tissue and plasma at the gene level. Considering the massive functions of MIF, we believe that during IOIP pathogenesis, this mighty cytokine could be playing an important role in IOIP disease development and maintenance.

**Keywords:** MIF, IOIP, pathogenesis, inflammation, tumorigenesis

## Introduction

Idiopathic Orbital Inflammatory Pseudotumor (IOIP) is recognized as an unspecific orbital inflammatory lesion, mimicking tumors with histological outcomes of inflammatory infiltration and tissue damage. In 1905, Birch-Hirschfeld described IOIP as an orbital neoplasm [1, 2]. The diagnosis of IOIP is usually by an exclusion method [3], because of its unknown pathogenesis. Before, IOIP was classified into the IgG4-related disease (IgG4-RD) [4], which had the features of inflammation and imitated the outcomes of malignant tumor [4, 5].

Macrophage migration inhibitory factor (MIF) was originally discovered as a soluble mediator secreted by activated T lymphoid cells [6]. MIF is secreted by both the immune and non-immune cells in response to many pathogens [7], and it plays an important role in autoimmune responses, infections, inflammation, tumorigenesis, etc. Extracellularly released MIF can stimulate the secretion of many other pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-1β, and trigger the expression of matrix metalloproteinase (MMP) [8-10]. Besides, MIF can enhance the recruitment of inflammatory cells, exacerbating inflammation, and tissue damage [11-13]. High level of MIF has been reported as a biomarker in the applications of critical and infectious illnesses and cancer [14-18].

Although an increasing body of evidence suggests that MIF may play a role in the biological activities related to inflammation-to-cancer transition [19], to date, the relationship between MIF-associated signaling pathways, orbital tumor (such as IOIP) pathogenesis and tumorigenesis has not been reported. In this study, we investigated a potential link between MIF and IOIP pathogenesis, in the development of inflammation, tissue proliferation, as well as the activation of downstream signaling pathways. Furthermore, our ongoing study indicated that MIF-induced Glucocorticoid resistance (GC) might contribute to the recurrent of GC therapy in IOIP patients. Therefore, treatment strategies for IOIP need to be reconsidered.

## Materials and methods

### Plasma and tissue samples

A total of 40 IOIP patients, along with 95 controls (including 76 normal donors and 19 patients with orbital cavernous hemangioma) were recruited from Beijing Tong Ren Hospital with approval of the local ethical committee. Plasma samples from all IOIPs and controls were assayed by serologic test. Tissue samples from 31 IOIP and 19 control group patients (i.e. cavernous hemangioma, CH) after immediately collection from surgical resection were analyzed in pathological examination.

Pretreatments: whole blood was centrifuged at 2,000 rpm for 10 min; the upper layer was then carefully removed into a clean tube and stored at -20°C; whole blood DNA was at last extracted from the left blood cells by using Gen Elute Blood Genomic DNA Kit (NA2010-1KT, Sigma, USA), and procedures were following the protocol strictly. Tissue samples were divided into two parts: one was embedded in paraffin and sectioned for pathological examination, and the other part was stored in liquid nitrogen for further extraction.

### Immunofluorescence (IF)

Incubate the slides at 70°C for 30 min, and then soak in xylene for 30 min to elute the paraffin. Dehydrate slides with sequential ethanol washes of 1 min each, starting with a 75%, followed by 80% and finishing with a 100% ethanol wash. Heat repair antigen for 30 min and cool the slides to room temperature, followed by 3 washes of 3 min each with phosphate buffer solution (PBS). Block non-specific binding sites with goat serum for 60 min before overnight incubation at 4°C with appropriate antibody. After extensive washing, slides were incubated for 20 min at 37°C with secondary antibody, and then washed 3 times in distilled water. Slides were then dehydrated in ascending grades of ethanol before clearing in xylene and mounting with a cover slip. IF related reagents were obtained from Zhongshanjinqiao Company (Beijing, China).

### Cytokine profiling

IOIP (n = 31) and CH (n = 19) tissue protein samples were tested with Bio-plex pro human Th17 cytokine panel (11 factors) (171-AA001M, Bio-rad, USA); IOIP (n = 32) and normal donor (n = 22) plasma samples were detected by 21 factors (MFO-005KMII, Bio-rad) and 27 factors (M50-OKCAFOY, Bio-rad, USA) cytokine panels. Cytokine test was strictly followed the protocols for the Bio-plex kits. Working flow was as followed: a. Prewet wells for filter plate. b. Add 50 µl 1 × beads to wells. c. Wash 2 × 100 µl. d. Add 50 µl standards, blank and samples, incubate at RT with shaking at 850 rpm. e. Wash 3 × 100 µl. f. Add 25 µl 1 × detection antibody, incubate 30 min at RT with shaking at 850 rpm. g. Wash 3 × 100 µl. h. Add 50 µl 1× streptavidin-PE, incubate 10 min at RT with shaking at 850 rpm. i. Wash 3 × 100 µl. j. Resuspend in 125 µl assay buffer, shake at 850 rpm for 30 sec. k. Read plate on Bio-plex 100 HTF system (Bio-Rad, USA).

### Enzyme-linked immunosorbent assay

Human MIF ELISA kit was purchased from RayBio. 96-well microplates were coated with capture antibody. Plasma samples and standards were then added and incubated for 2 hours. After washing, HRP-conjugated detection antibody was added into each well. Plates were washed three times, followed by stopping the enzyme reaction with stop solution. The optical densities of each well were read in 30 min at 450 nm using a micro-plate reader (PerkinElmer, USA).

### Western blotting

Take 100 mg from each tissue sample in liquid nitrogen and extract whole protein following protocol of the kit (T-PER78510, Thermo Scientific, USA). Measure concentration of each
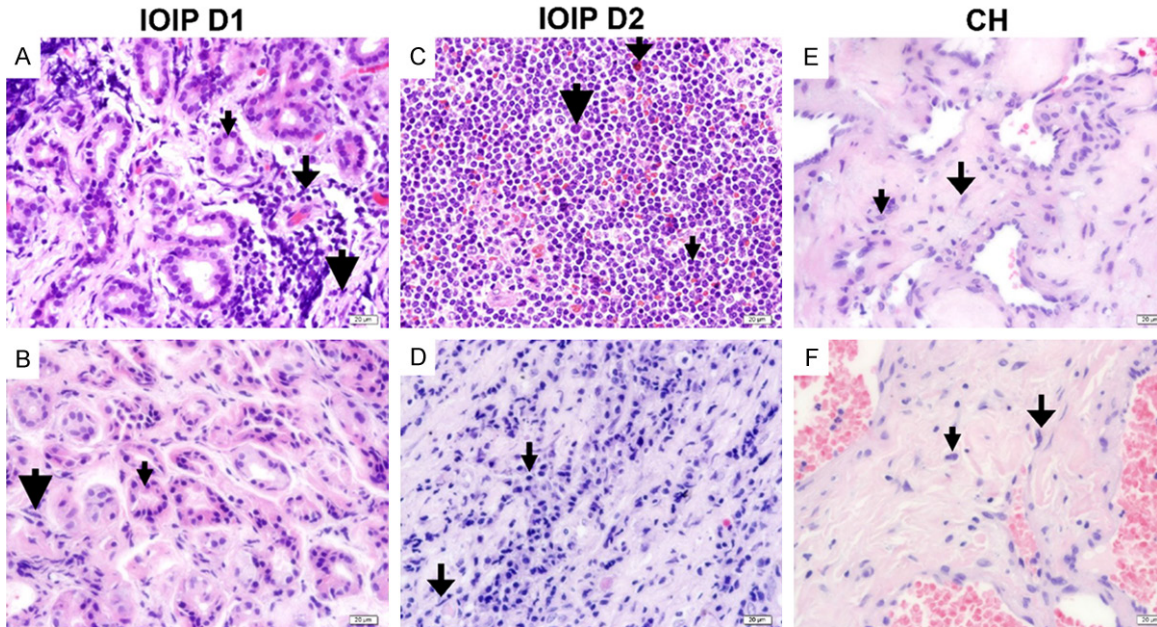
**Figure 1.** Histological analysis of the tissue paraffin sections of the IOIP and CH groups ($n_{IOIP}$ = 4, $n_{CH}$ = 2). A and B. Representative H&E staining of the IOIP D1 group patients characterized by typical cell types including glandular epithelium cell proliferation (small arrows), different degrees of lymphocytes infiltration (medium arrow) and fibrosis (large arrows). C and D. H&E staining of the IOIP D2 group patients characterized by massive lymphocytes infiltration (small arrows), different levels of fibrosis (medium arrow in D), eosinophils (medium arrow in C) and macrophages (large arrow) visible in the affected tissue. E and F. Histological features of the CH group patients (control group) containing few lymphocytes (small arrow) and extensive fibrosis (medium arrow). Original magnification 40X.

protein sample, and then take 30 μg from each sample to mix with equal volume of 2× loading buffer (containing SDS). Boil each tissue lysate at 100°C for 5 minutes and aliquot so as to reduce and denature.

Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight markers. Run the gel for 30 min at 60 V and another 1 to 2 hours at 100 V, and then protein samples were transferred to nitrocellulose membranes. Membranes blocked for 1 hour at 37°C and incubated with primary antibody overnight at 4°C. After washing in PBS and 0.5% Tween20 (PBST) for 3 times, 10 min each and the membranes were incubated with the secondary antibody (fluorescence labeled). Membrane was washed again in PBST and at last soaked in PBS for another 3 min before scanned in Odyssey scanner.

*Genotyping of MIF-173G>C and MIF-794 CATT[5-8] repeat polymorphisms*

The polymerase chain reaction (PCR) of MIF-173G>C contained 1 ng of genome DNA (i.e. whole blood DNA), 12.5 μl Tag PCR Master Mix (Qiagen, USA), 10 μmoles of both forward and reverse primers (forward 5'-TTG-CAC-CTA-TCA-GAG-ACC-3', reverse 5'-TCC-ACT-AAT-GGT-AAA-CTC-G-). Target length is 445 bp. PCR of MIF-794 CATT[5-8] contained 1 ng of genome DNA (i.e. whole blood DNA), 12.5 μl Tag PCR Master Mix, 10 μmoles of both forward and reverse primers (forward 5'-TGC-AGG-AAC-CAA-TAC-CCA-TAG-G-3', reverse 5'-AAT-GGT-AAA-CTC-GGG-GAC-3'). Target length is 346 bp. Reaction conditions are: 95°C for 12 min, followed by 40 cycles of 95°C for 30 sec, 58°C for 30 s and 72°C for 40 sec, the last segment is 72°C for 10 min.

PCR products were then purified and controlled into a total amount of 1 μg-2 μg, which were all diluted in ddH$_2$O, and reverse primers of 5 pmole/μl were prepared for genotyping. Luminahiseq 2000 was used for genotyping of MIF-173G/C and MIF-794 CATT[5-8] polymorphisms which was performed by Sangon Biotechs Company (Shanghai, China).

*Microarray analysis*

Fresh tissue samples from 21 IOIPs and 9 CHs were subjected to Phalanx One Array® Gene Expression Profiling (Taiwan) following the stan-
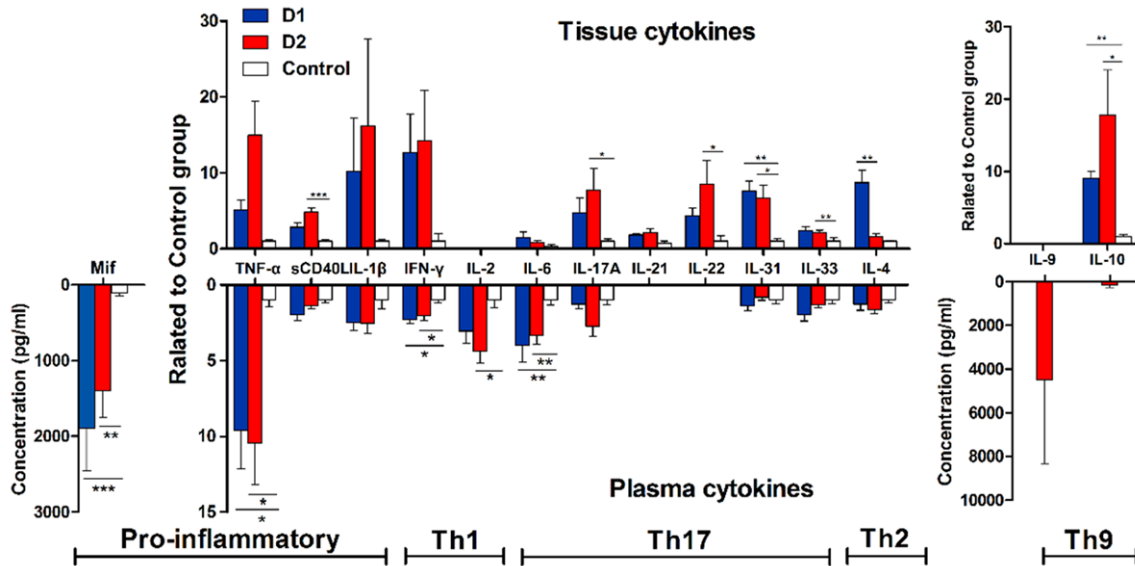
**Figure 2.** Cytokine profiles in tissue and plasma of IOIP D1 and D2 patients. Cytokine profiles are categorized by pro-inflammatory and 4 kinds of immune types (Th1, Th2, Th17 and Th9), and displayed in IOIP D1 and D2 groups. Controls for tissue cytokines profile are samples from CH patients; controls for plasma cytokines profile are samples from normal donors. Tissue cytokine profile: $n_{D1} = 12$, $n_{D2} = 19$, $n_{Control} = 19$; plasma cytokine profile: $n_{D1} = 13$, $n_{D2} = 19$, and $n_{Control} = 22$. Bars show means ± SE; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

dard protocol in the Affymetrix Gene Chip Expression Analysis Technical Manual. Micro-array data was analyzed using Bx Genomic DB system (BioInfo Rx, Inc., Madison, WI). Clustering analysis and heat map creation were performed using dChip software (Genome Biol. 2001; 2(8), PMID: 11532216).

*Antibodies*

Antibodies used in IF and WB assays were: Mouse anti human p53 (ab26, abcam, USA), Rabbit anti human bcl-2 (ab7973, abcam, USA), Rabbit anti human MIF (ZS-201210, Zhongshan-Jinqiao, China), Mouse anti human CD74 (NBP2-29465, Novus Biologicals, USA), Rabbit anti human CXCR4 (NB100-56437), Rabbit anti human Phospho-p38 MAP Kinase (p-p38MAPK) (Thr180/Tyr182) (#9211, Cell Signaling, USA), Rabbit anti human Phospho-Glucocorticoid Receptor (p-GR) (Ser211) (#4161, Cell Signaling, USA), Rabbit anti NF-κB p65 (ZS-1090, ZhongshanJinqiao, China) and Rabbit anti NF-κB p50 (ZS-114, ZhongshanJinqiao, China).

*Statistical analysis*

Statistical analysis was performed using SPSS version 18.0 software (SPSS Inc, Chicago, IL) and Graphpad prism 5 (Graph Pad Software

Inc, La Jolla, CA). The difference of IgG4 positive rate between IOIPs and the controls was assessed by Chi-square test. Differences of cytokine, gene profile and plasma MIF between IOIPs and control groups were analyzed by unpaired T test. All of the differences were considered statistically significant at $P<0.05$. Hardy-Weinberg genetic balance between IOIPs and controls was tested for MIF-173G/C and MIF-794CATT[5-7] ($0.1>P>0.05$), showing the frequency of these genotypes was in a state of genetic equilibrium.

**Results**

*Histological features of IOIP*

Thirty seven IOIP tissue samples were stained with hematoxylin and eosin (HE). Based on the HE staining results, IOIPs were classified into 2 groups following the standard: affected tissue in/out of the lacrimal gland. HE staining of IOIP (**Figure 1**) showed that Disease type 1 (D1) (n = 15) represented patients with affected lesion in lacrimal gland, where large amounts of lacrimal gland epithelial cells were observed; patients with affected tissue out of lacrimal gland, characterized by massive lymphocyte infiltration and different degrees of fibrosis, were grouped as Disease type 2 (D2) (n = 22).
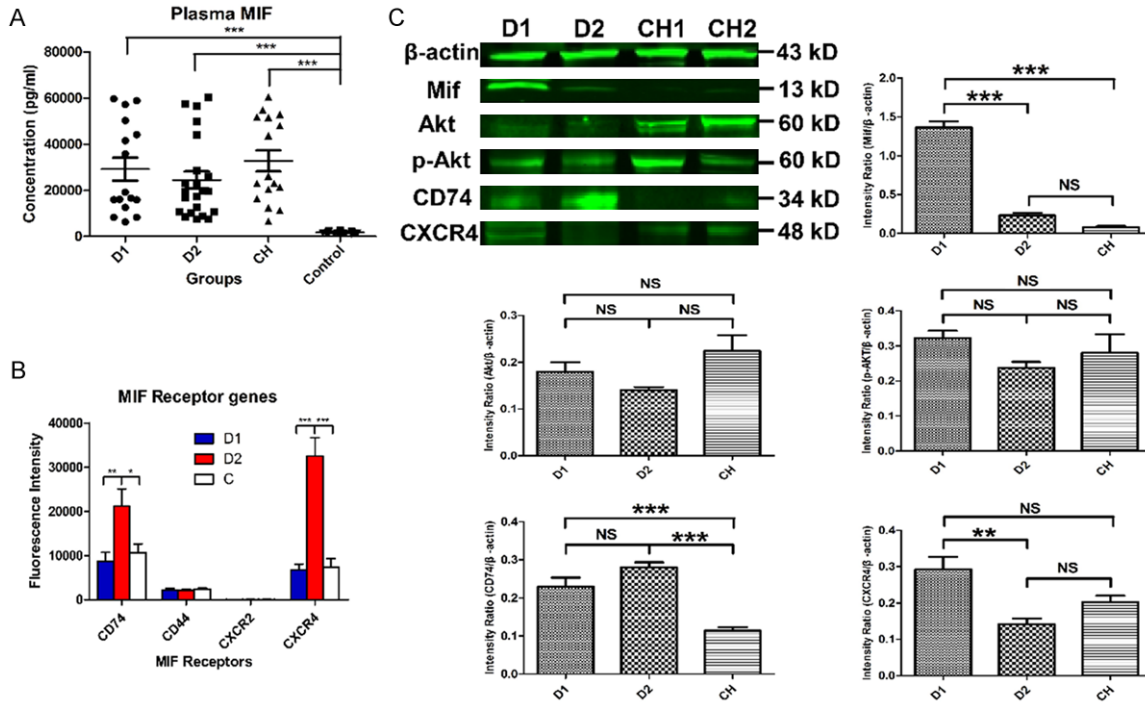
**Figure 3.** Strong expression of MIF and 2 of its receptors at the mRNA and protein levels out of IOIP (D1 and D2 group) and CH patients as well as AKT signaling pathway activation. MIF expression in the plasma of IOIP and CH (control) patients, as well as normal people (negative control, NC) (A); $n_{D1}$ = 16, $n_{D2}$ = 22, $n_{CH}$ = 16, $n_{NC}$ = 20. Gene expression level 4 of MIF's receptors was detected by using microarray analysis (B); $n_{D1}$ = 13, $n_{D2}$ = 8, $n_{CH}$ = 9. Western blot analysis of MIF, receptor CD74 and CXCR4, AKT and p-AKT (C) are displayed; $n_{D1}$ = 5, $n_{D2}$ = 5, $n_{CH}$ = 4. Bars show means ± SE; p-AKT, phospho-Akt; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

The following results were all sorted in accordance with the 2 groups of D1 and D2.

*Tissue and plasma cytokine profiles in IOIP*

In order to investigate the immune and inflammatory involvement in IOIP, 48 cytokines in the plasma and 15 cytokines in the tissue were tested. As shown in **Figure 2**, MIF was the most up-regulated cytokine in the plasma, with higher concentration (>10000 pg/mL) in both D1 and D2 groups. Further analysis of MIF in the plasma was performed to expand the sample amount. Using ELISA, the concentration of MIF in D1, D2, CH, and normal controls (health donor) was calculated. As shown in **Figure 3A**, MIF in D1, D2 and CH groups were all significantly higher than the control group ($P<0.001$, 95% CI 22540-34190; IOIP: Mean = 30060± 4785 pg/mL; Normal Donor: Mean = 1700±63 pg/mL)
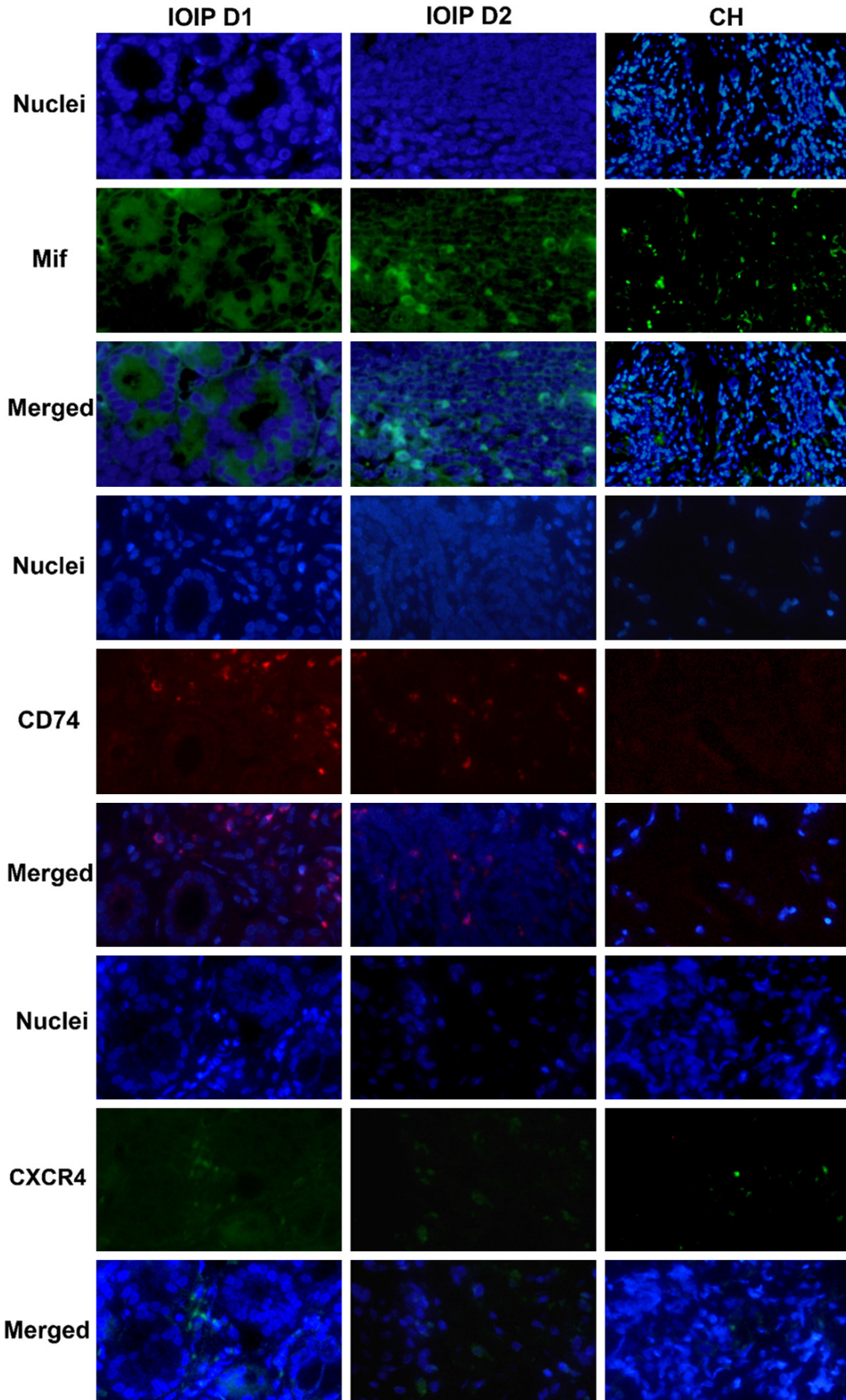
D1 and D2 exhibited significantly higher levels of TNF-α in both the plasma and the tissue. Another cytokine in the TNF family, sCD40L and pro-inflammatory factor IL-1β were also up-regulated in D1 and D2. Th1 cell related cytokines (IFN-γ and IL-2) were elevated in D2 and D1. Cytokines essential for Th9 and Treg differentiation were also expressed in both D1 and D2, especially in the D2 group. IL-10 was significantly higher in both tissue and plasma of IOIP compared to the control group, especially in D2. Among the Th17 related cytokines, IL-6 and IL-17 were significantly up-regulated in the plasma of both D1 and D2 groups. In the affected tissues, other Th17 cytokines, including IL-17A, IL-21, IL-22, and IL-31 were much higher in both of the groups.

*Expression of MIF and its receptors in the tissue*

As shown in **Figure 3**, MIF and its receptors were also detected in the tissue due to its high expression in the cytokine profile of plasma. Microarray analysis for the gene expression of 4 MIF receptors are shown in **Figure 3B**, among which *CD74* ($P<0.05$) and *CXCR4* ($P<0.001$)

**Figure 4.** Immunofluorescence staining for MIF (FITC, green), CD74 (H&L, red) and CXCR4 (FITC, green) in IOIP (D1 and D2) and CH groups. Original magnification, 40X.
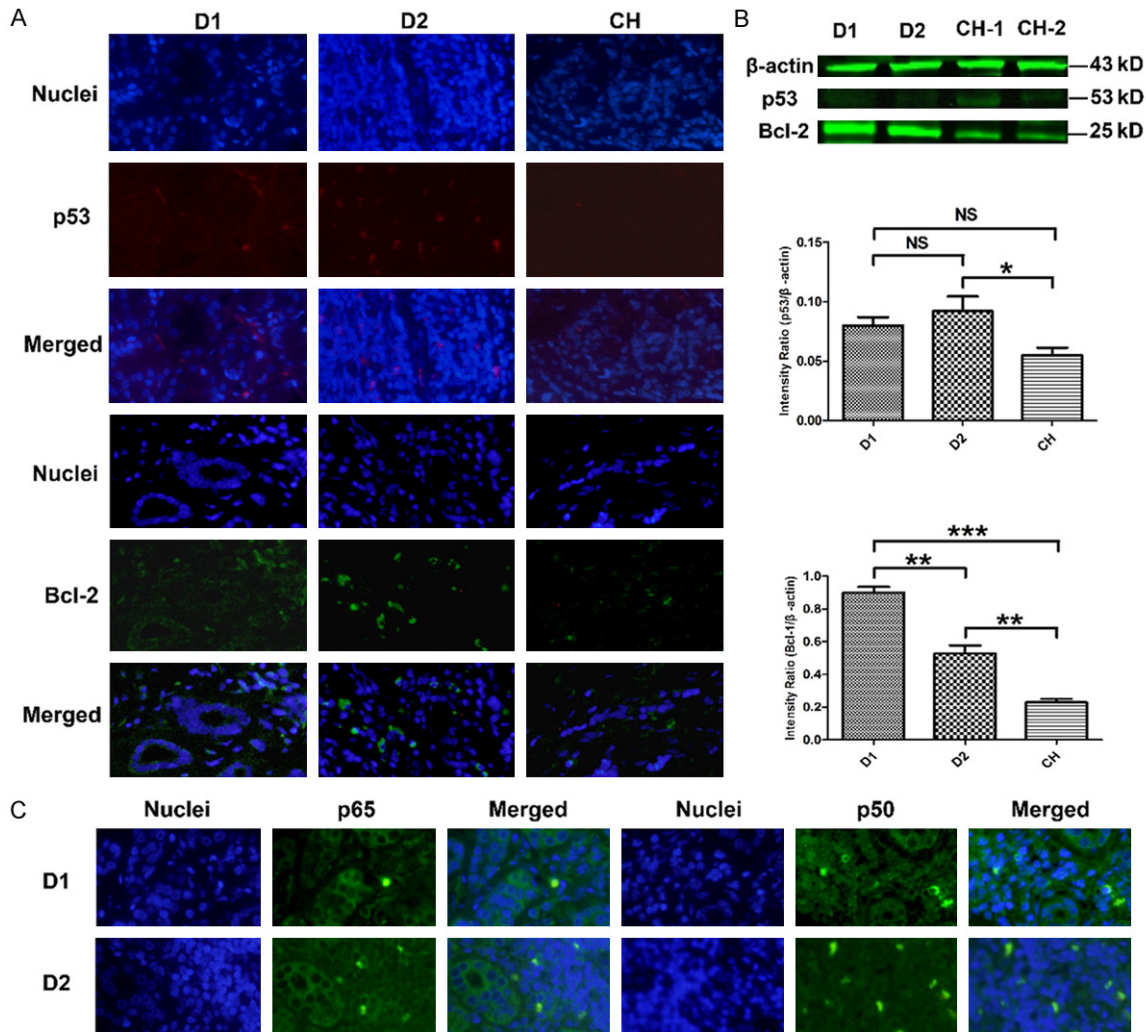


**Figure 5.** AKT downstream protein expression in IOIP (D1 and D2) and CH groups. (A) shows the immunofluorescence for p53 (H&L, red) and bcl-2 (FITC, green) proteins in IOIP and CH. Original magnification: 40X. Western blot analysis of p53 and bcl-2 in IOIP and CH groups is shown in (B) for confirmation. The expression of NF-κB monomers p65 and p50 was detected by immunofluorescence and shown in (C). Original magnification: 40X.

were elevated in D2 group compared to CH group. Western blot (WB) analysis indicated that MIF was expressed in D1 (*P*<0.05) and D2 (ns) groups compared with CH group (**Figure 3C**). The ratios of CD74/β-actin and CXCR4/β-actin were higher in IOIP group, especially in D1, though not statistically significant. We also performed immunofluorescent staining with anti-MIF (FITC, green), CD74 (H&L, red) and CX-CR4 (FITC, green) in both groups of IOIP. Immunofluorescent analysis confirmed the results of the WB, where MIF, CD74, and CXCR4 dis-

played positive fluorescence in the lacrimal gland epithelial cells and lymphocytes of D1, and only in the lymphocytes of D2 (**Figure 4**).

*Activation of AKT and downstream signaling pathways*

MIF induces AKT related signaling pathways, which can further activate many downstream cascades, such as inflammation and tumorigenesis. Therefore, in this study, we tested some important proteins involved in the activation of these pathways.
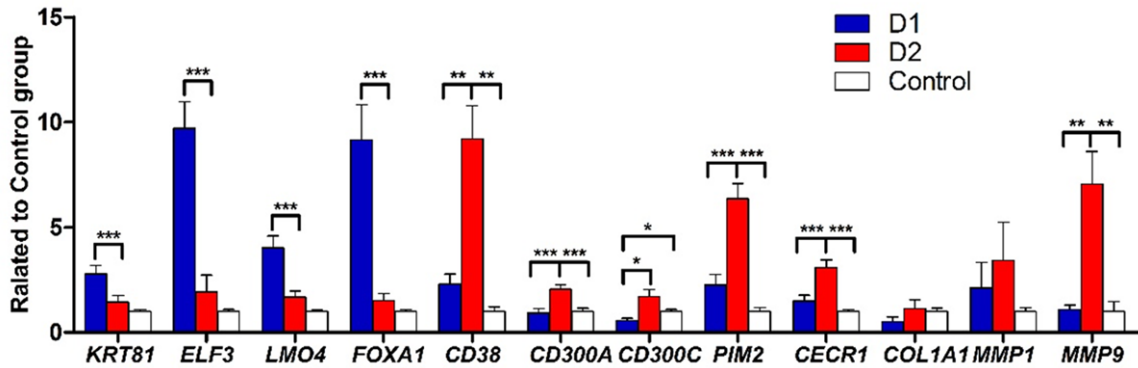
**Figure 6.** Microarray analysis for the tumor related gene expression in the 2 IOIP groups. Control: RNA samples from CH patients. Bars show means ± SE; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

WB analysis for cytoplasmic phosphorylated AKT (p-AKT) showed higher expression in both the groups in IOIP compared to AKT protein (**Figure 3C**). These results proved the activation of the AKT signaling pathway in IOIP and CH groups. Mutant and wild type p53 expression displayed weak positive in D1 and D2 of IOIP, while a stronger positive in CH group (**Figure 5B**). WB analysis demonstrated significantly stronger expression of Bcl-2 in the D1 and D2 groups than in the CH group ($P<0.01$) (**Figure 5B**). Immunofluorescent detection of bcl-2 and p53 confirmed the results of the WB, showing stronger positive staining in both D1 and D2 than CH group (**Figure 5A**). Immunofluorescent analysis of the 2 monomers of NF-κB (**Figure 5C**) demonstrated nuclear location, indicating activation of NF-κB.

*Genotyping of MIF-173G>C and MIF-794 CATT$^{5-8}$ repeat polymorphisms*

Genotyping of the MIF-173G>C polymorphism of MIF in IOIP patients (n = 40) and healthy individuals (n = 72) revealed no significantly different frequencies of genotypes and haplotypes in both the groups. However, the ratio of IOIP patients carrying the MIF-173C allele was higher than that of the control group (26.25% VS 15.28%). Similarly, no significant differences of MIF-794 CATT$^{5-8}$ repeat polymorphisms were seen between IOIP (n = 37) and control group (n = 37). The carrier ship of MIF-794 CATT$^7$ in IOIP showed more frequency than in controls (17.51% VS 8.11%), whereas MIF-794 CATT$^6$ in IOIP demonstrated less frequency than in controls (39.19% VS 52.70%).

*Upregulated tumor-related genes in D1 and D2*

The results of the microarray analysis for D1, D2 and CH are shown in **Figure 6**. The mRNA levels of *KRT81, ELF3, LMO4* and *FOXA1*, representing epithelial concretization and cancer, appeared significantly higher in D1 group than in D2 ($P<0.001$) and CH ($P<0.001$) groups. Transcript levels of *CD38, CD300A, CD300C, PIM2, Col1A1, CECR1, MMP1,* and *MMP9*, which are cancer markers, disease severity index, or autoimmune diseases-related genes, were higher in the D2 group. Among all these genes, only *CD38* (P<0.05), CD300A (P<0.001), CD300C (P<0.01), PIM2 (P<0.001), CECR1 (P<0.001) AND MMP9 (P<0.05) showed significant differences between D1 and D2.

Discussion

IOIP was first described in 1905, however, the pathogenesis and etiology of the disease still remains elusive. Our understanding of this disorder has been complicated due to its wide spectrum of clinical and histological presentations. Notably, IOIP has the features of both tumor and inflammation, indicating that this disease might be in the intermediate stage of tumor and inflammation. Our data indicated that MIF was the most up-regulated cytokine in the plasma. In this study, we reported the important role of MIF in IOIP inflammation and cytokine expression, as well as the downstream signaling pathways. Finally, we also tested the single nucleotide polymorphisms (SNPs) of MIF gene expression in order to reveal the mechanism of MIF elevation in IOIP pathogenesis at the genetic level.
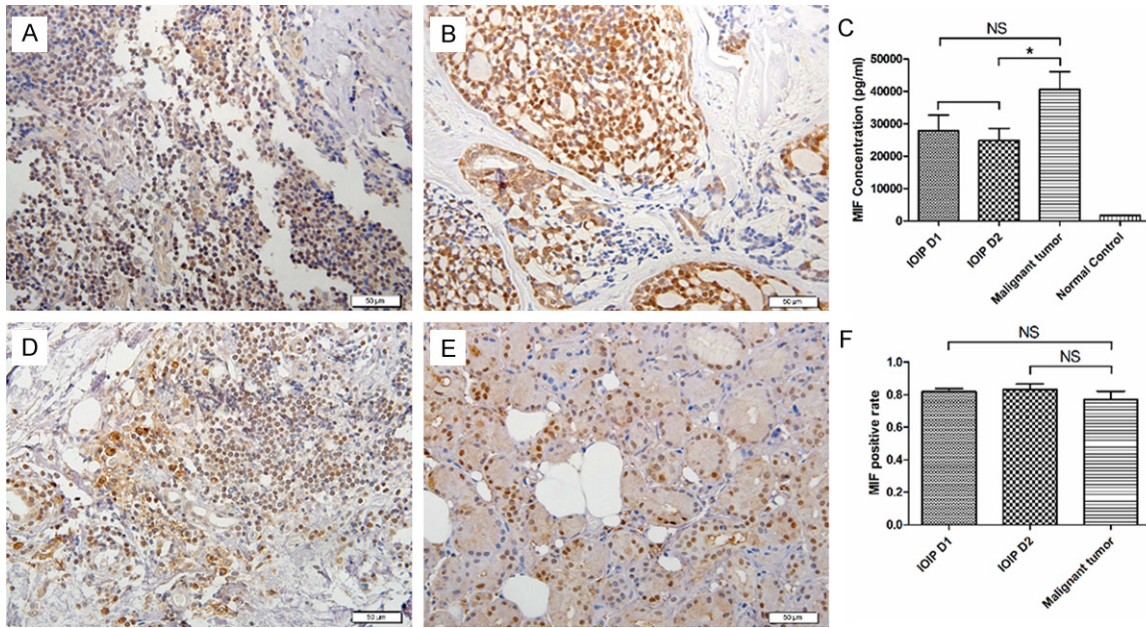
**Figure 7.** Comparison of MIF expression between IOIP subtypes and malignant orbital tumors. A-D. Representative IHC results of MIF in orbital MLEL, orbital B lymphoma, lacrimal adenoid cystic carcinoma, and mixed tumor of lacrimal gland, respectively, which all belong to malignant orbital tumor. E. Displayed serological MIF level in two groups of IOIP, malignant orbital tumors and normal control group. F. Summarized the positive rate of MIF IHC in IOIP and orbital malignant tumor group. Original magnification 40X.

*MIF and cytokine expression*

As one of the pro-inflammatory cytokines, MIF has been identified as a key factor in the inflammation responses. In our study, other pro-inflammatory cytokines besides MIF, such as TNF-α, IL-1β and IL-6 were all highly expressed in IOIP tissue and/or plasma (**Figure 2**). Elevated levels of the representative cytokines suggested that different immune responses were involved in D1 and D2, including Th2 in D1 (IL-4 in tissue), Th1 in D2 (IL-2 and IL-25 in tissue), Th17 in D1 and D2 (IL-6, IL-17, IL-21, etc. in plasma and tissue), Th9 and Th10 in D2 (IL-9 and IL-10 in plasma and tissue). Based on these results, we hypothesize that the immune response in IOIP is a network, in which Th17 may play an important role in IOIP's pathogenesis. We are currently investigating this hypothesis in a separate study.

Since MIF lies in the upstream location in the events leading to dysregulated immune inflammatory responses that cause autoimmune diseases, it has been implicated in the pathogenesis of multiple organ-specific autoimmune diseases including type 1 diabetes, rheumatoid arthritis, multiple sclerosis, Guillain-Barr'e syn-drome, Crohn's disease, autoimmune myocarditis, glomerulonephritis, hepatitis, thyroiditis, and psoriasis [20]. Another upstream cytokine TNF-α, as well as its family member sCD40L, were significantly elevated (P<0.01) in the tissue cytokine profile. It has been reported that MIF induces the secretion of TNF by macrophages, and consecutively, TNF augments MIF production [21]. Wijbrandts et al. reported that anti-TNF-α therapy might not be sufficient for rheumatoid arthritis (RA) patients. Sustained downregulation of MIF can be used as a potential new mechanism to reduce vascular inflammation, and perhaps also cardiovascular morbidity in RA patients [22]. Therefore, MIF in cooperation with other cytokines such as TNF-α may influence the downstream pro-inflammatory factors, and further induce massive immune responses in IOIP.

*MIF and AKT pathway*

MIF is an important pro-inflammatory cytokine that not only induce the secretion of other pro-inflammatory factors, but also activate massive downstream responses and activities, including cell proliferation, chemokine expression, anti-apoptosis, inflammation, integrin activa-

**Table 1.** MIF-173 G/C genotype detection in whole blood DNA of IOIP and Control groups

| | IOIP | | Control | |
|---|---|---|---|---|
| | No. | % | No. | % |
| MIF-173 Genotype | | | | |
| G/G | 21 | 52.50 | 51 | 70.83 |
| G/C | 17 | 42.50 | 19 | 26.39 |
| C/C | 2 | 5.00 | 2 | 2.78 |
| Total | 40 | 100.00 | 72 | 100.00 |
| MIF-173 Allele | | | | |
| G | 59 | 73.75 | 121 | 84.03 |
| C | 21 | 26.25 | 23 | 15.97 |
| Total | 80 | 100.00 | 144 | 100.00 |

**Table 2.** MIF-794 CATT repeats detection in whole blood DNA of IOIP and Control groups

| | IOIP | | Control | |
|---|---|---|---|---|
| | No. | % | No. | % |
| MIF-794 Genotype | | | | |
| 5/5 | 4 | 10.81 | 4 | 10.81 |
| 5/6 | 20 | 54.05 | 19 | 51.35 |
| 5/7 | 4 | 10.81 | 2 | 5.41 |
| 6/6 | 2 | 5.41 | 8 | 21.62 |
| 6/7 | 5 | 13.51 | 4 | 10.81 |
| 7/7 | 2 | 5.41 | 0 | 0.00 |
| Total | 37 | 100.00 | 37 | 100.00 |
| MIF-794 Allele | | | | |
| 5 | 32 | 43.24 | 29 | 39.19 |
| 6 | 29 | 39.19 | 39 | 52.70 |
| 7 | 13 | 17.57 | 6 | 8.11 |
| Total | 74 | 100.00 | 74 | 100.00 |

tion, etc. via many pathways [23]. In these functions of MIF, CD74 is an essential receptor. Relying on receptors CD74, CXCR2/4, and/or CD44, MIF can activate downstream pathways such as ERK, NF-κB, PI3K-AKT, JNK/AP1, etc.

In our data, CD74 was elevated in the tissue at both mRNA and protein levels. The expression of CXCR4, another important MIF receptor, was higher in the D2 group. Phosphorylated AKT, mutant p53, bcl-2, as well as p65 and p50 monomers were all detected in the affected tissue of D1 and D2 groups, representing the activation of AKT, p53, BCL and NF-κB pathways, respectively. Several studies have reported the potential role of MIF between accumulated inflammation and cancer growth [19]. Therefore, we hypothesized that in IOIP, MIF induces con-

tinual activation of downstream AKT and NF-κB pathways by taking advantage of its receptors-CD74 and CXCR4. These activities of MIF and the following responses cause chronic and accumulated inflammation in the affected tissue, thereby leading to a trend of deterioration, i.e. tumorigenesis [24, 25]. On the other hand, MIF can suppress apoptosis by directly inhibiting p53 activity or enhancing bcl-2 function, eventually contributing to tumorigenesis [26].

Inflammation and cancer have long been linked together. In clinical trials of several cancers, MIF has been used as one of the potent biomarkers [27, 28]. Mechanistic studies revealed that MIF stimulates fibroblast cells in vitro and activates ERK-MAPK pathway [29], thereby leading to subsequent cell proliferation. Numerous studies have shown that MIF is a crucial factor that forms a microenvironment favorable for the transition of inflammation to tumorigenesis. AKT and downstream signaling pathways (e.g. p53, BCL, NF-κB, et al.) may play functional roles in the formation of this microenvironment. However, further studies investigating the role of MIF in the development of malignancies are needed. As shown in **Figure 6**, although the genes up-regulated in D1 and D2 were different due to different location of the affected tissue, most of the highly expressed genes were cancer-associated. This, to some degree indicated towards the existence of possible tumorigenesis in both the groups, and that MIF was an important candidate inducer.

*MIF in malignant orbital tumors*

We also evaluated the expression of MIF in malignant orbital tumors. Interestingly, most of the patients who underwent orbital malignant tumors showed elevated serological and histological MIF (**Figure 7**), including mixed lacrimal gland tumor and orbital lymphoma. These results suggest that MIF also plays a role in these tumors, and it could be a missing link between IOIP and malignant orbital tumor. Our ongoing research is focused on the role of MIF at the cellular level.

*Gene expression of MIF*

Human MIF gene is located on chromosome 22q 11.2 [30]. The SNPs at position 173 (MIF-173G/C) and CATT5-8 tetra nucleotide repeat
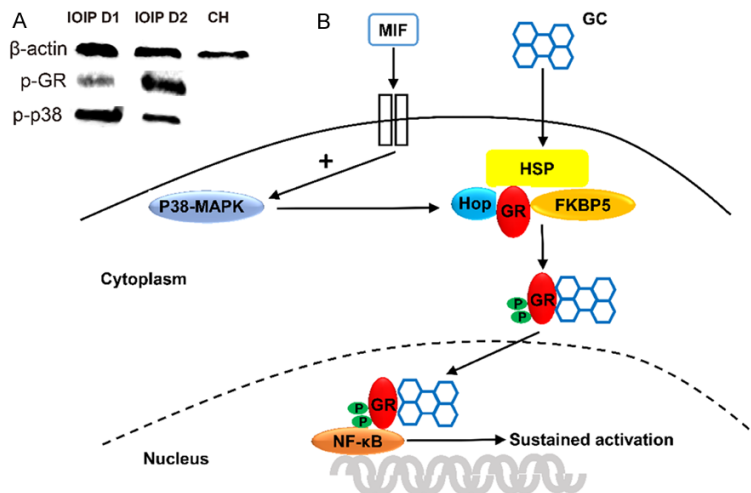
**Figure 8.** Potential GC resistance network involved in IOIP. A. Showed WB test of p-GR and p-p38 expression in IOIP D1 and D2 subgroups. B. Representative the potential signal pathway of GC resistance in IOIP according to the gene and protein expression profiles.

element starting at position 794 in the promoter region of the MIF gene have been shown to play a role in the inflammatory conditions [31]. In a study on RA, an autoimmune and inflammatory disease, high levels of circulating MIF were found, and genetically determined by MIF-173C, MIF-794 CATT7and CATT8 [31].

In our DNA sequencing results (**Tables 1** and **2**), IOIP SNPs of -173C and -794 CATT7 alleles accounted proportions of 26.25% and 17.57%, respectively, compared to those in the control group of 15.97% and 8.11%, respectively. Contrarily, -794 CATT6 in IOIP shared 39.19%, much less than the controls of 52.10%. Although no significant differences were found due to a small sample size, the ratios in IOIP were much higher than in control group, which provided one possible explanation for high expression level of MIF in both blood and tissue in IOIP. In addition to this, based on different proportions of the 2 SNPs in IOIP and control group, MIF-173 C and -794 CATT7 alleles might have added a genetic component to IOIP risk, or contributed to the pathogenesis of IOIP, while -794 CATT6 might be a protective factor.

*Glucocorticoid (GC) resistance*

GC is one of the effective medicines for IOIP patients [32, 33], and one of its functions is preventing the activation of NF-κB. However, some studies have found that GC treatment had a recovery rate of only 37%, and 50% of the

IOIP patients had to undergo recurring treatment [34]. MIF exhibits potent anti-GC effect, and has been shown to be associated with some inflammatory diseases [35]. MIF is induced by GC, which then inhibits their anti-inflammatory effects [36]. Our results suggested that p-GR and p-p38-MAPK were elevated in both groups of IOIP (**Figure 8A**). Nuclear expression of p65 and p50 in both IOIP subtypes, indicated the activation of NF-κB (**Figure 5C**). Taking all of these together, we suppose MIF might lead to GC-resistance in IOIP by continuously activating the p38-MAPK and NF-κB pathway [37] (**Figure 8B**), and our ongoing study is focusing on the mechanism at the primary cell level.

In conclusion, this is the first study to demonstrate significantly elevated expression of MIF and its receptors in IOIP. Similarly, downstream pro-inflammatory factors, cytokines, and typical proteins in several signaling pathway activation were all highly expressed. Considering the massive role of MIF, this mighty cytokine may continuously activate certain signaling pathways such as AKT and NF-κB in IOIP, thereby causing chronic inflammation, tumorigenesis and cancer development. This mechanism can also influence other malignant orbital tumors such as mixed lacrimal gland tumor and orbital lymphoma, due to high serological and histological MIF expression in these patients. Genetic alleles of MIF-173C and MIF-794 CATT7 might add further risk to IOIP pathogenesis. Besides, MIF also contributes to GC-resistance in traditional IOIP therapy, therefore, reconsideration of IOIP treatment may be needed in the future. Further investigations studying the role of MIF in IOIP pathogenesis and proliferation are required.

## Disclosure of conflict of interest

None.

## Authors' contribution

Pengxiang Zhao: experimental operation, manuscript writing and review, data analysis and statistics. Jianmin Ma: surgery and sample supply. Lei shang: experimental operation and data collection. Adzavon Yao Mawulikplimi: experimental operation and sample processing. Xiaoyue Wang: experimental operation and sample processing. Yanan Wu: experimental operation. Fei Xie: manuscript review. Linqi Yang: experimental operation. Danying Chen: Data collection. Xuemei Ma: team work organization, data analysis, and manuscript review.

Address correspondence to: Dr. Xuemei Ma, College of Life Science and Bio-engineering, Beijing University of Technology, Beijing 100124, P. R. China. Tel: +8613810276409; Fax: +8601067392001; E-mail: xmma@bjut.edu.cn

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