Correlations of MIF polymorphism and serum levels of MIF with glucocorticoid sensitivity of sudden sensorineural hearing loss

Wen-Yan Zhu, Xin Jin, Yong-Chi Ma and Zhi-Biao Liu

Abstract
Objective: This study explored the relationship between macrophage migration inhibitory factor (MIF) gene polymorphism (−173G/C) and glucocorticoid sensitivity in sudden sensorineural hearing loss (SSNHL).

Methods: A total of 120 patients with SSNHL were divided into a glucocorticoid-sensitive group and a glucocorticoid-resistant group. A group of 93 healthy individuals served as the control group. Serum MIF levels of the participants were measured and MIF genotyping was performed.

Results: The frequency of the MIF −173C allele was significantly higher in glucocorticoid-sensitive patients than in glucocorticoid-resistant patients. Serum MIF levels were significantly higher in SSNHL patients than in healthy controls, and higher in the glucocorticoid-sensitive group than in the glucocorticoid-resistant group of SSNHL patients, which was unexpected. Compared with patients with the GG genotype, patients with the −173C allele (GC and CC genotypes) had significantly higher levels of serum MIF and superoxide dismutase activity and lower levels of tumor necrosis factor-α and malondialdehyde.

Conclusion: The MIF −173G/C polymorphism is associated with glucocorticoid sensitivity in SSNHL patients. The C allele can result in higher MIF production, reduced oxidative stress, and greater glucocorticoid sensitivity.
Keywords
Sudden sensorineural hearing loss (SSNHL), macrophage migration inhibitory factor (MIF),
glucocorticoid, polymorphism, glucocorticoid sensitivity, oxidative stress

Introduction
Sudden sensorineural hearing loss (SSNHL) is defined as a decline in hearing of at least 20 dB in two adjacent frequencies that occurs suddenly within 72 hours. The patient’s hearing may be completely or partially restored or it may not be restored, resulting in permanent hearing loss. At present, glucocorticoid is the first-line treatment of SSNHL worldwide. However, numerous studies have suggested that some SSNHL patients have reduced sensitivity to glucocorticoid therapy because of glucocorticoid resistance. Therefore, glucocorticoid resistance may be a clinical treatment bottleneck of SSNHL; in patients with reduced steroid sensitivity, much higher doses of steroids might be a way to enhance steroid sensitivity and achieve a better therapeutic effect.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine that was initially found to inhibit migration of macrophages after its release from T lymphocytes. MIF is a widely recognized regulator of the innate immune response, and it promotes expression and production of proinflammatory mediators such as interleukin (IL)-1β and IL-6. Therefore, it is not surprising that MIF is involved in the pathogenesis of a variety of acute and chronic inflammatory diseases, such as asthma, rheumatoid arthritis, sepsis, diabetes, and atherosclerosis. Paradoxically, after treatment of patients with glucocorticoids, MIF is released into the supernatant of cultured macrophages and T lymphocytes. In fact, MIF counter-regulates the action of glucocorticoid and inhibits release of prostaglandin E2, leukotriene B4, and IL-6 from immune cells stimulated by glucocorticoids. Thus, MIF negatively regulates the anti-inflammatory effects of glucocorticoids in various inflammatory diseases. Furthermore, administration of recombinant MIF has been shown to inhibit the suppressive action of glucocorticoids on proinflammatory cytokine production.

The inhibitive effect of MIF on glucocorticoids leads to individual variability in response to glucocorticoids because of a single nucleotide polymorphism (SNP) in the MIF gene, specifically a change in the promoter region (−173) that changes a guanine (G) to cytosine (C). The −173C allele is reported to be associated with higher mRNA expression and circulating levels of MIF and more severe radiologic joint damage in patients with rheumatoid arthritis. Some studies have shown that the MIF −173G/C gene polymorphism may increase the risk of glucocorticoid resistance in childhood nephrotic syndrome and nephrotic syndrome. Therefore, this SNP may also affect glucocorticoid sensitivity in SSNHL and thus could act as an index of glucocorticoid response and be used to guide individualized treatment.

In this study, we evaluated the association of the −173G>C MIF polymorphism with glucocorticoid sensitivity of SSNHL patients. To achieve this, we measured serum MIF and tumor necrosis factor-α.
(TNFα) levels, performed genotyping at the −173G > C polymorphism of MIF, and determined the auditory threshold, thus dividing patients into a glucocorticoid-sensitive group and a glucocorticoid-resistant group. To evaluate oxidative stress, we measured serum malondialdehyde (MDA, a lipid peroxidation product of polyunsaturated fatty acids) content and superoxide dismutase (SOD, an antioxidant enzyme) activity in SSNHL patients.

Methods

Study population

We recruited 120 patients with SSNHL between January 2015 and June 2017 in the Department of Otolaryngology Head and Neck Surgery of our hospital, and included 69 men and 51 women with a median age of 57 years. A total of 93 age- and sex-matched healthy subjects without a history of hearing loss, other ear disorders, or autoimmune diseases were selected as the control group, including 58 men and 35 women (median age of 53 years). The selection criteria were based on the Sudden Sensorineural Hearing Loss Diagnosis and Treatment Guidelines published by the Chinese Medical Association Archives of Otolaryngology Head and Neck Surgery Branch. The diagnostic standards were as follows: SSNHL occurring in a few minutes, hours, or within 3 days; nonfluctuating sensorineural hearing loss, sensorineural hearing loss of 20 dB or more over three contiguous audiometric frequencies; unknown cause due to systemic or local factors; accompanied by tinnitus, ear blockage sensation, and non-recurrent dizziness, nausea, and vomiting; and no other cranial nerve injury with the exception of eighth cranial nerve injury. The exclusion criteria were hearing loss caused by a negative brain and internal auditory canal magnetic resonance imaging (MRI) scan, such as multiple sclerosis, trauma, drug, noise, or surgery complications. No patients received treatment for hearing loss before taking the hearing test. All patients were administrated a methylprednisolone (Pfizer, New York, NY, USA) intravenous drip within 8 hours of admission to hospital according to the following regimen: 80 mg/day for 4 days, 40 mg/day for 3 days, and 20 mg/day for 3 days.

The study was performed in accordance with the Declaration of Helsinki (1964), and the study protocol was approved by the Institutional Review Board of our hospital. The study was supported by Ethics Committee of The Affiliated Huaian No. 1 People’s Hospital of Nanjing Medical University. Written informed consent was signed by each participant.

Audiometry examination

Auditory threshold was measured by pure tone audiometry before treatment (initial), once every 2 days during treatment, and 1 month after treatment for final evaluation. Audiometry was evaluated using four frequencies (0.5, 1, 2, and 3 kHz). Auditory improvement was evaluated using the following formula: threshold reduction = initial threshold − final threshold. An average threshold reduction of >15 dB was considered an improvement and the patient was regarded as glucocorticoid sensitive; otherwise, the patient was regarded as glucocorticoid resistant.

Genotyping of MIF gene

Genomic DNA was extracted from peripheral blood mononuclear cells of SSNHL patients and healthy individuals. Primers of the rs755622 SNP were synthesized by Shanghai GeneCore BioTechnologies Co. Ltd. (Shanghai, China). The forward primer was 5′-ACTAAGAAAGACCGAGGC-3′ and the reverse primer was 5′-GGGGCACGTTGGTGTTTAC-3′.
These primers were designed to amplify a 366-bp segment of the promoter region. The PCR was carried out in a volume of 25 μL. The reaction conditions of PCR were initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. PCR products were confirmed by agarose gel electrophoresis and then sent to Shanghai Hybio Biotechnology Co. Ltd. (Shanghai, China) for sequencing.

**ELISA for serum levels of MIF and TNFα**

Venous blood samples were collected from each participant (patients and controls) within 24 hours of admission to the hospital. To evaluate systematic inflammation in SSNHL patients, serum MIF and TNFα levels were measured by using commercial Quantikine ELISA kits (MIF: DMF00B; TNFα: DTA00D; R&D Systems, Minneapolis, MN, USA), and their values were determined by measuring optical density at 450 nm using a microplate reader. The serum MIF and TNFα values of each participant were calculated from a standard curve using their recombinant human proteins, respectively.

**Measurement of serum SOD and MDA**

To evaluate the oxidative extent of SSNHL patients, serum malondialdehyde (MDA) content and superoxide dismutase (SOD) activity levels were determined using UV spectrophotometric colorimetry. Activity of SOD was examined using the xanthine oxidase method and expressed as units per milliliter. Content of MDA was determined using the thiobarbituric acid method (Nanjing Institute of Biological Engineering, Nanjing, China), and expressed in millimoles per milliliter.

**Statistical analysis**

The IBM SPSS statistical software package (version 19.0 IBM Corp., Armonk, NY, USA) was applied to analyze the data. Continuous variables were expressed as medians (interquartile ranges) and analyzed by Wilcoxon–Mann–Whitney test. Categorical variables were expressed as frequencies (percentages) and analyzed by χ² test or Fisher’s exact test. Spearman’s rank correlation tests were used to analyze correlations between serum MIF and glucocorticoids inhibition rate. \( P < 0.05 \) was considered to indicate a statistically significant difference.

**Results**

**MIF genotype is associated with response to glucocorticoid in SSNHL patients**

The profiles of the SSNHL group and the control group are shown in Table 1. No significant differences in age or sex were found between the SSNHL group and the control group. The auditory threshold [median (interquartile range)] was significantly higher in the SSNHL group [34.8 (31.3–37.0) dB] than in the control group [13.6 (12.4–15.0) dB] (\( P < 0.001 \)). The SNP rs755622 conformed to Hardy-Weinberg equilibrium in the control and SSNHL groups (both \( P > 0.05 \)). In the SSNHL group, 84 (70%) patients had the homozygous GG genotype, 31 (25.8%) patients had the heterozygous GC genotype, and 5 (4.2%) patients had the homozygous CC genotype. Genotype frequencies did not differ between patients and controls.

We investigated the association between MIF gene polymorphism and hearing recovery after treatment with glucocorticoid in SSNHL patients. According to the threshold reduction, 74 patients were regarded as glucocorticoid sensitive (threshold reduction >15 dB), and 46 patients were...
regarded as glucocorticoid resistant (threshold reduction ≤15 dB). The initial auditory threshold was significantly higher in the glucocorticoid-resistant group than in the glucocorticoid-sensitive group. This might indicate that patients with significantly poorer hearing at onset are less likely to show a glucocorticoid response. There was a significant difference in the frequency of genotypes (CC, GC, GG) of MIF –173C between glucocorticoid-sensitive and glucocorticoid-resistant patients \((P = 0.021; \text{ Table 2})\). Moreover, compared with glucocorticoid-resistant patients,
glucocorticoid-sensitive patients had a significantly higher frequency of the C allele ($P = 0.006$; Table 2).

**Associations of serum MIF with MIF (−173 G/C) gene polymorphism**

The serum MIF level was significantly higher in SSNHL patients (median 66.5 ng/mL) than in healthy controls (median 29.8 ng/mL) ($P < 0.001$; Figure 1a). The 120 SSNHL patients were divided into two groups according to their responsiveness to glucocorticoids. Patients who were sensitive to glucocorticoids had higher serum MIF levels than patients who were resistant to glucocorticoids ($P = 0.001$; Figure 1b). The 120 SSNHL patients and 93 healthy controls were divided into two groups according to the presence of the −173C allele and evaluated for serum MIF levels. There was no significant difference in serum MIF level between healthy controls with and without the −173C

![Figure 1](image-url)

**Figure 1.** Effect of the MIF −173C allele on serum levels of MIF. Serum MIF levels were significantly higher in SSNHL patients than in healthy controls (a), and significantly higher in glucocorticoid-sensitive SSNHL patients than in glucocorticoid-resistant patients (b). No significant difference in serum MIF levels was detected between patients with the C allele (GC and CC genotypes) and without the C allele (GG genotype) in healthy controls (c). SSNHL patients with the C allele (GC and CC genotypes) had significantly higher serum MIF levels than patients lacking the C allele (GG genotype) (d). Wilcoxon–Mann–Whitney test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are presented as boxplots, where the box represents 50% of the values, the bold black line indicates the median per group, horizontal lines show minimum and maximum values of the calculated non-outlier values, and open circles indicate outlier values. MIF, macrophage migration inhibitory factor; SSNHL, sudden sensorineural hearing loss.
allele ($P > 0.05$; Figure 1c). However, SSNHL patients carrying the $MIF -173C$ allele had significantly higher serum MIF levels than SSNHL patients without the $-173C$ allele (i.e., the GG genotype) ($P = 0.009$; Figure 1d).

**Associations of $MIF (-173C)$ allele with inflammation and oxidative stress**

We then investigated the effect of $MIF -173C$ allele on pathological indicators of SSNHL patients. Patients carrying the $MIF -173C$ allele had a significantly better (lower) auditory threshold at presentation than patients without C allele (the GG genotype) ($P = 0.003$; Figure 2a). The $MIF -173C$ allele was associated with higher inflammation, as evidenced by higher serum TNF$\alpha$ in patients carrying the $MIF -173C$ allele than those without the C allele ($P = 0.031$; Figure 2b). The $MIF -173C$ allele might also have some inhibitory effect on oxidative stress. Compared with patients with the $MIF$ GG genotype, patients carrying the $MIF -173C$ allele

![Figure 2.](image)

Figure 2. Effect of the $MIF -173C$ allele on inflammation and oxidative stress. Compared with SSNHL patients without the C allele (GG genotype), SSNHL patients with the C allele (GC and CC genotypes) had significantly lower final auditory thresholds (a), significantly higher serum TNF$\alpha$ levels (b), significantly lower serum MDA content (c), and significantly lower SOD activity (d). Wilcoxon–Mann–Whitney test: *$P < 0.05$, **$P < 0.01$. Data are presented as boxplots, where the box represents 50% of the values, the bold black line indicates the median per group, horizontal lines show minimum and maximum values of the calculated non-outlier values, and open circles indicate outlier values. MIF, macrophage migration inhibitory factor; SSNHL, sudden sensorineural hearing loss; TNF$\alpha$, tumor necrosis factor $\alpha$; MDA, malondialdehyde; SOD, superoxide dismutase.
had significantly lower serum MDA content ($P = 0.012$) and higher SOD activity ($P = 0.001$; Figure 2c, 2d).

**Discussion**

In this study, we investigated the association of the $-173G > C$ MIF polymorphism with glucocorticoid sensitivity in patients with SSNHL. Serum MIF levels were significantly higher in the glucocorticoid-sensitive group than in the glucocorticoid-resistant group of SSNHL patients. Compared with patients with the GG genotype, patients with the $-173C$ allele (GC and CC genotypes) had significantly lower auditory thresholds, higher concentrations of serum MIF and TNF$\alpha$, higher SOD activity, and lower levels of MDA. Thus, $-173G > C$ may be associated with glucocorticoid sensitivity of SSNHL patients.

The MIF $-173G > C$ mutation is increasingly recognized as a cause of immune system disorders and it increases the risk of glucocorticoid resistance. Here, we found opposite results in the association of $-173C$ with higher glucocorticoid sensitivity in SSNHL patients. Our results are in accordance with another report, in which the frequency of MIF $-173C$ allele carriers was significantly elevated in SSNHL patients who responded to glucocorticoid treatment compared with those who did not respond.20 Our results are in contrast to other reports that the $-173C$ allele and MIF promote glucocorticoid resistance in various inflammatory diseases.13 MIF itself is an inflammatory cytokine that has the potential to aggravate hearing loss, as shown by higher serum TNF$\alpha$ in SSNHL patients with the $-173C$ allele. TNF$\alpha$ plays a key role in the pathophysiology of sensorineural hearing loss. However, SSNHL patients with the $-173C$ allele also had higher SOD activity and lower MDA content, indicating an inhibitory effect on oxidative stress, which might offset the aggravating effect on hearing loss. In fact, although it has been reported that the MIF $-173C$ allele increases glucocorticoid resistance in various diseases, this association was not observed in patients with nephrotic syndrome, childhood acute lymphoblastic leukemia, or idiopathic thrombocytopenic purpura.21–23 These discrepant results suggest varying roles of MIF in the inflammatory cascade in different disorders in ways that variably influence the anti-inflammatory response to glucocorticoids. Although MIF is elevated in inflammatory processes and higher, prolonged increases in MIF are correlated with steroid resistance, higher MIF levels in this study were correlated with improved steroid responsiveness.

The increased glucocorticoid response of the MIF $-173C$ allele and corresponding high MIF production might also be explained by salutary intracochlear roles of MIF. MIF can promote the growth of primary axons from the spiral auditory ganglion to the inner ear and maintain the survival of neurons. MIF$^{-/-}$ knockout mice are impaired in hearing and have a reduced number of sensory hair cells.24 MIF is highly expressed in the mouse inner ear and MIF$^{-/-}$ knockout mice show abnormal inner ear morphology and accelerated age-related hearing loss.25 MIF can also promote the survival of cochlea and perivascular-resident macrophage-like melanocytes from the stria vascularis and prevent the loss of the cochlear hair cells; the mechanism of MIF may be related to activation of the PI3K/Akt signaling pathway.26–28 Indeed, MIF shows survival effect in cells other than those of the inner ear. In myocardial ischemia/reperfusion injury, MIF reduced apoptosis and provided cardioprotection by inhibiting oxidative stress and the apoptosis-inducing c-Jun N-terminal kinase (JNK) pathway in the myocardium.29,30 Moreover, MIF can induce AMPK activation and promote
glucose uptake during ischemia/reperfusion, thereby protecting the heart via metabolic pathways.\textsuperscript{31,32} Our previous study showed that MIF protected cochlear cells from oxygen-glucose deprivation (OGD)-induced injury.\textsuperscript{33} Therefore, the present study showed that SSNHL patients with the $-173C$ allele had higher MIF levels and, considering the apparent protective role of MIF in the cochlea, also had improved cochlear steroid responsiveness.

This study demonstrated higher serum MIF levels in SSNHL patients with the $-173C$ allele than in those without. This indicates that the presence of the mutant C allele can promote binding of transcription factors, resulting in a significantly increased MIF expression. However, healthy individuals with the $-173C$ allele had only slightly increased serum MIF, with no significant difference from healthy individuals lacking the $-173C$ allele. Our data showed that MIF levels are higher in SSNHL patients than in healthy control individuals. In addition, patients in the steroid-sensitive group appeared to have higher MIF levels than those in the steroid-resistant group, and overall responsiveness to steroids was increased by the $-173C$ allele. As SSNHL is an inflammatory disease, MIF protein may be induced in SSNHL, as evidenced by a significant positive correlation between serum MIF and TNF$\alpha$.\textsuperscript{34} Furthermore, MIF expression is induced by glucocorticoids, and a positive correlation between circulating MIF levels and steroid dosage has been reported.\textsuperscript{35} Meanwhile, exogenous administration of recombinant mouse MIF significantly enhanced serum corticosterone concentration in rats.\textsuperscript{36} Whether this positive feedback between MIF and glucocorticoids underlies the increased glucocorticoid response of the $MIF$ $-173C$ allele and higher serum MIF remains unknown and deserves further study.

Our study showed that MIF might have an inhibitory effect on oxidative stress, as shown by lower serum MDA content and higher SOD activity in SSNHL patients carrying the $MIF$ $-173C$ allele. Thus, MIF might promote hearing recovery by inhibiting oxidative stress. The cochlea is an organ with high aerobic metabolism and it produces a large amount of reactive oxygen species (ROS). Under normal circumstances, active oxygen in the cochlea and the antioxidant defense system maintain a dynamic balance. However, when stimulated by ischemic hypoxia, noise, or ototoxic drugs, ROS generated in the cochlea surpass the removal capacity of the antioxidant system, causing oxidative damage to the cochlea and progressive hearing loss and deafness.\textsuperscript{37} MDA results from lipid peroxidation of polyunsaturated fatty acids and is a marker for oxidative stress. SOD is an important antioxidant defense enzyme that catalyzes the superoxide radical (O$_2^-$) into either ordinary molecular oxygen (O$_2$) or hydrogen peroxide (H$_2$O$_2$). In patients with noise-induced hearing loss, blood concentrations of MDA are increased, whereas antioxidant enzyme activity of SOD is decreased.\textsuperscript{38} Oxidative stress is the common pathological mechanism of noise-induced hearing loss and SSNHL, as manifested by the presence of significantly elevated serum ROS in SSNHL patients.\textsuperscript{39} The addition of antioxidative therapy concurrently with glucocorticoid treatment can effectively improve the degree of hearing recovery in patients with SSNHL.\textsuperscript{40} This indicates that oxidative stress is not only involved in the pathogenesis of SSNHL but is also an effective target for SSNHL treatment. MIF protects cells from oxidative insult and has been identified as a specific binding protein of BTZOl, an antioxidant response element (ARE) activator. MIF protein binds to BTZOl and then protects cells and organs from oxidative insults via ARE activation in animal
models with oxidative stress such as ischemia/reperfusion injury and inflammatory bowel diseases. Therefore, patients carrying the MIF –173C allele might increase MIF production and thus protect against oxidative damage to the cochlea, as manifested by increased glucocorticoid sensitivity.

The limitations of the present study are as follows. This was a retrospective study and thus patients were not under strict control, so bias is inevitable. For example, the glucocorticoid-resistant group had significantly higher initial auditory thresholds than the glucocorticoid-sensitive group. This might mean that patients with severe inner ear damage are less likely to respond to glucocorticoid treatment. Therefore, we compared the auditory threshold reduction, rather than the final auditory threshold, between the two groups. Nevertheless, the fact that glucocorticoid-sensitive patients had a lower mean of hearing threshold remains a potential confounder. Second, MIF seems to have variable (opposite) effects on glucocorticoid response. Whereas MIF shows a cochleoprotective effect that mitigates cochlea damage and enhances the therapeutic effect of glucocorticoid, MIF is also a proinflammatory cytokine and might antagonize the effect of glucocorticoid. The balance between these two effects may determine the final effect of MIF in SSNHL patients. In our study, the beneficial effect of MIF on the cochlea exceeded its proinflammatory effect, which confirmed our previous study, in which MIF promoted cochlear cell survival after oxygen-glucose deprivation-induced injury. The antagonistic effect of MIF on glucocorticoid response should be further investigated in detailed and precise experiments. In addition, the expression and production of MIF by SNP rs755622 needs to be explored in an in vitro study, and functional analysis of downstream pathway of MIF in SSNHL patients is warranted.

In conclusion, the MIF –173C allele may enhance the glucocorticoid sensitivity of SSNHL, increase serum MIF levels, and demonstrate an antioxidative effect by enhancing MIF production. Although high MIF levels are associated with autoimmune disorders and nonresponsiveness to glucocorticoids, the reverse seems to be true in steroid responsive of SSNHL, and MIF might show survival and antioxidative effects in glucocorticoid-sensitive patients. Increasing the MIF content in the cochlea of SSNHL patients or animals may protect the cochlea and promote hearing recovery in SSNHL patients.

Authors' contributions
Wen-Yan Zhu designed the study, performed experiments, and wrote the manuscript; Xin Jin performed experiments; Yong-Chi Ma provided statistical analysis; and Zhi-Biao Liu revised the manuscript.

Availability of data and material
Data supporting the results of this study are available from the corresponding author on reasonable request.

Declaration of conflicting interest
The authors declare that there is no conflict of interest.

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