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# Polymorphism and expression of macrophage migration inhibitory factor does not contribute to glucocorticoid resistance in idiopathic thrombocytopenic purpura

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Glucocorticoids (GCs) are considered the important drugs used in treatment of idiopathic thrombocytopenic purpura (ITP). However, about 10–30% patients with ITP develop GC resistance after standard treatment with GC. The macrophage migration inhibitory factor (MIF) has been shown to act as a counter-regulator of the anti-inflammatory and immunosuppressive effects of GCs on immune cells. In addition, MIF-173G/C polymorphism was associated with higher MIF expression both *in vitro* and *in vivo*. In this case-control study, we investigated the association of GC resistance with MIF polymorphism and expression. MIF mRNA expression was analyzed by semiquantitative real-time RT-PCR in GC-sensitive and GC-resistant ITP patients. MIF protein expression in serum was performed by ELISA. Genotyping for the MIF -173G/C polymorphism was analyzed by a Polymerase chain reaction Tm-shift genotyping method. We found no association of GC resistance and MIF mRNA and protein expression. According to sex, age, initial blood platelet count, and disease course, the patients were further subdivided into 8 groups, no statistical difference was found. In addition, we compared the distribution of the MIF -173G/C genotype and allele frequencies between the GC-sensitive ITP patients and the GC-resistant and found no statistical difference. The present study suggested that MIF polymorphism and expression does not contribute to GC resistance in ITP.

## 1. Introduction

Idiopathic thrombocytopenic purpura (ITP) is considered as an organ specific autoimmune disorder characterized by a severely decreased platelet number and mucocutaneous bleeding. Incidence of ITP is reported to be around 3 per 100000 people per year in Europe (Cines and Blanchette 2002; Cines and Bussel 2005). The pathogenesis of ITP is not fully understood. It is thought that ITP associated platelets are opsonized by autoantibodies and prematurely destroyed by the reticuloendothelial system (El-Shiekh et al. 2012; Beardsley and Ertem 2005; Kuwana et al. 2003).

Glucocorticoids (GCs) are widely used in the treatment of ITP (Schipperus and Fijnheer 2011). GCs action leading to attenuation of relapse symptom is attributed to the effects of decreasing the accelerated platelet destruction by FcR blockade/activating and immunosuppression and enhancing platelet production (Crow et al. 2006; Siragam et al. 2006; Cines et al. 2009). However, GCs are not effective in all patients with ITP, and about 10–30% patients with ITP develop GC resistance after three to six months of standard treatment with GC (Cines et al. 2009).

The macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine produced by T-cells, macrophages and other inflammatory cells (Calandra and Roger 2003). MIF is expressed in a wide range of endothelial cells, eosinophils, epithelial cells, lymphocytes, macrophages and neutrophils (Stosic-Grujicic et al. 2009). MIF has been shown to act as a counter-regulator of the anti-inflammatory and immunosuppressive effects of GCs on immune cells (Denkinger et al. 2004). Furthermore, recent studies demonstrated that MIF may play a role in the development of glucocorticoid resistance in patients with rheumatoid arthritis, systemic lupus erythematosus, atherosclerosis, sepsis and acute respiratory distress syndrome (Stosic-Grujicic et al. 2009). In addition, the study has been shown that MIF-173G/C polymorphism was associated with higher MIF expression both *in vitro* and *in vivo*. Vivarelli et al. (2007) found that the -173*C allele may represent a predictor of poor response to intra-articular glucocorticoid treatment In patients with oligoarticular juvenile idiopathic arthritis.

However, so far, there are no reports evaluating the polymorphism and expression of MIF in GC-sensitive and GC-resistant ITP patients. In this case-control study, we investigated the association of GC resistance with MIF polymorphism and expression in this case-control study.

## 2. Investigations and results

### *2.1. Demographic and clinical characteristics of the ITP patients*

After steroid treatment, no significant differences were observed between the GC-sensitive and GC -resistant groups (Table 1).

**Table 1: Distribution of clinicopathological feaures of ITP patients, shown for both case –control groups**

| | GC-sensitive (n = 84) | GC-resistant(n = 70) | P value |
|---|---|---|---|
| Sex | | | |
| Male | 33 | 26 | 0.78 |
| Female | 51 | 44 | 0.35 |
| Age at diagnosis | | | |
| <14 years | 37 | 29 | 0.84 |
| >14 years | 47 | 41 | 0.86 |
| Initial PLT(/ul) | | | |
| $<10 \times 10^9$ | 25 | 20 | 0.92 |
| $>10 \times 10^9$ | 59 | 50 | 1 |
| Disease course | | | |
| Acute | 22 | 25 | 0.35 |
| Chronic | 62 | 45 | 0.58 |

### *2.2. MIF mRNA expression in the GC-sensitive and GC-resistant patients*

We compared the MIF mRNA expression between the total GC-resistant ITP and the total GC-sensitive patients, and found no statistical difference [median (range): $28.71 \pm 5.97$ vs. $27.19 \pm 6.30$, $P > 0.05$; Table 2]. According to sex, age, initial blood platelets count, and disease course, the patients were further subdivided into 8 groups, We compared the distribution of the MIF mRNA expression between each of these 4 groups and the controls, respectively. No statistical difference was found (Table 3).

### *2.3. Serum MIF protein expression in the GC-sensitive and GC-resistant patients*

We compared the MIF protein expression between the total GC-resistant ITP and the total GC-sensitive patients, and found no statistical difference [median (range): $12.82 \pm 5.54$ vs. $13.96 \pm 6.15$, $P > 0.05$; Table 2]. We compared the MIF protein expression between each of these eight subgroups and the controls respectively. No statistical differences were found (Table 4).

### *2.4. Genotyping for the MIF MIF-173G/C polymorphism*

The allelic frequencies of G and C in the GC-resistant ITP patients were 82.9% and 17.1%, respectively (Table 5). The distribution of GG, GC and CC genotypes in the GC-resistant was 67.8%, 29.1% and 2.8%, respectively. The allelic frequencies of G and C in the GC- sensitive ITP patients were 84.5% and 15.5%, respectively. The distribution of GG, GC and CC genotypes in the GC- sensitive was 71.4%, 26.1% and 2.4%, respectively. No significant differences were observed in MIF -173G/C polymorphism between the GC-sensitive ITP patients and the GC-resistant patients ($P = 0.92$, OR, 0.80; CI, 0.11–5.89 for genotypes and $P = 0.81$, OR, 0.89; CI, 0.48–1.62 for T allele).

**Table 2: Median mRNA and protein expression of MIF shown for the total group of patients and for the two GC reponder groups**

| | Median MIF RNA expression[a] | P | Median MIF protein expression (ng/ml) | P-value |
|---|---|---|---|---|
| Total group | $27.89 \pm 6.11$ | | $13.36 \pm 6.34$ | |
| GC response | | | | |
| GC-sensitive | $27.19 \pm 6.30$ | >0.05 | $12.82 \pm 5.54$ | >0.05 |
| GC- resistant | $28.71 \pm 5.97$ | | $13.96 \pm 6.15$ | |

[a] Normalized to GADPH.

**Table 3: Median mRNA expression of MIF in subgroups of ITP patients**

| | N | Median MIF mRNA expression[a] | | P-value |
|---|---|---|---|---|
| | | GC- sensitive | GC- resistant | |
| Sex | | | | |
| male | 59 | $26.85 \pm 7.02$ | $29.62 \pm 6.15$ | >0.05 |
| female | 95 | $27.44 \pm 6.15$ | $28.26 \pm 6.10$ | >0.05 |
| Age at diagnosis | | | | |
| <14 years | 66 | $25.69 \pm 6.12$ | $27.66 \pm 5.76$ | >0.05 |
| >14 years | 88 | $28.32 \pm 6.45$ | $29.55 \pm 5.98$ | >0.05 |
| Initial PLT(/ul) | | | | |
| $<10 \times 10^9$ | 45 | $23.64 \pm 3.71$ | $25.16 \pm 3.44$ | >0.05 |
| $>10 \times 10^9$ | 109 | $28.61 \pm 6.42$ | $30.08 \pm 5.68$ | >0.05 |
| Disease course | | | | |
| acute ITP | 47 | $30.57 \pm 3.11$ | $31.25 \pm 4.54$ | >0.05 |
| chronic ITP | 107 | $26.14 \pm 6.72$ | $27.43 \pm 7.63$ | >0.05 |

[a] Normalized to GADPH

## 3. Discussion

Although GC therapy is the treatment of choice for ITP, some patients fail to adequately respond to high doses of GC therapy. Three possible mechanisms of GC resistance have been proposed: reduced numbers of glucocorticoid receptors (GRs), altered affinity of the ligand for GRs and reduced ability of the GRs to bind the DNA (Wansheng et al. 2012).

It has been demonstrated that the correlation between increased MIF level and GC resistance has been reported in several inflammatory diseases and in human CEM T-cell lines (Flaster et al. 2007; Leng et al. 2009). A recent study showed enhanced MIF expression in colonic mononuclear cells from patients with glucocorticoid-resistant ulcerative colitis and the MIF antibody restores the anti-inflammatory response to GCs in these cells (Ishiguro et al. 2006). In addition, it was reported that elevated MIF levels in sepsis patients play an important role in the development of GC resistance, and that the NO signaling pathway is not involved in this pathological process (Jing and

**Table 4: Protein expression of MIF in subgroups of ITP patients**

| | N | Median MIF Protein expression (ng/ml) | | P-value |
|---|---|---|---|---|
| | | GC- Sensitive | GC- Resistant | |
| Sex | | | | |
| male | 59 | $12.82 \pm 6.97$ | $13.78 \pm 7.63$ | >0.05 |
| female | 95 | $12.57 \pm 6.45$ | $14.06 \pm 5.69$ | >0.05 |
| Age at diagnosis | | | | |
| <14 years | 66 | $13.35 \pm 6.92$ | $14.42 \pm 7.09$ | >0.05 |
| >14 years | 88 | $12.71 \pm 6.48$ | $14.66 \pm 5.56$ | >0.05 |
| Initial PLT(/ul) | | | | |
| $<10 \times 10^9$ | 45 | $13.02 \pm 5.79$ | $14.55 \pm 5.28$ | >0.05 |
| $>10 \times 10^9$ | 109 | $12.29 \pm 7.34$ | $13.07 \pm 6.21$ | >0.05 |
| Disease course | | | | |
| acute ITP | 47 | $11.98 \pm 6.91$ | $12.55 \pm 5.80$ | >0.05 |
| chronic ITP | 107 | $13.45 \pm 6.00$ | $15.24 \pm 5.19$ | >0.05 |

Bu 2011). Furthermore, Wang et al. (2012) reported that higher MIF levels have been found in serum and PBMCs of steroid-resistant SLE patients compared with steroid-sensitive patients, and that MIF may play a role in the formation of steroid resistance in SLE by affecting the NF-kB/IkB signaling cascade. These results suggest that MIF expression may be implicated in GC resistance.

In the present study, we compared MIF mRNA expression in PBMCs from patients with the GC-sensitive and GC-resistant, and no statistical difference was found. Moreover, analysis of serum MIF protein expression showed no major difference between the GC-sensitive and GC-resistant patients. Even subgroup analysis including sex, age, and initial blood platelet count, the disease course showed no statistically significant difference in MIF mRNA and protein expression of the GC-sensitive and GC-resistant groups. Compared with previous reports, our results suggest that MIF expression should not be associated with GC resistance in ITP. Due to the small sample size, our findings are preliminary and need further assessment in larger case-control studies.

Although the exact mechanisms by which MIF expression is associated with GC resistance remain largely unknown, Griga et al. (2007) reported polymorphisms of the MIF gene which lead to a constitutively higher MIF expression associated with glucocorticoid resistance in patients with Crohn's or inflammatory bowel disease (Griga et al. 2007). we evaluated the effects of the MIF -173G/C polymorphism in the GC-sensitive and the GC-resistant ITP patients. We observed no significant differences in the MIF -173G/C genotypes and alleles between the GC-sensitive ITP patients and the GC-resistant patients. Similar to our results, Ziino O et al. (2005) reported that the MIF-173G/C polymorphism did not contribute to GC resistance in childhood acute lymphoblastic leukemia (Ziino et al. 2005). Our result concerning the MIF -173G/C polymorphism influence on GC sensitivity in ITP by comparing previous studies were inconclusive and controversial, these differences may arise from the environmental and ethnic differences between the populations in the collected samples.

In conclusion, this is the first attempt to correlate the response to GC with polymorphism and expression of MIF in ITP patients. However, several limitations need to be considered in this study. Firstly, since selection of the SNP based on MIF -173G/C single nucleotide polymorphism (SNP) we cannot fully exclude the possibility that other SNPs located somewhere within the MIF gene locus are also associated with GC therapy. Secondly, the exact mechanisms by which MIF do not induce GC resistance in ITP need to be explored in future studies. Nevertheless, the present study suggests that MIF polymorphism and expression does not contribute to GC resistance in ITP.

**Table 5: MIF -173G/C polymorphism in the GC-sensitive and the GC-resistant ITP patients**

| | GC-sensitive n = 84 | GC-resistant n = 70 | P value | Odds ratio(95%CI) |
|---|---|---|---|---|
| | | MIF | | |
| Genotype | Frequency | | | |
| GG | 60(71.4%) | 48(67.8%) | | |
| GC | 22(26.1%) | 20(29.1%) | | |
| CC | 2(2.4%) | 2(2.8%) | | 1 |
| GC+CC | 24 (28.5%) | 22(31.9%) | 0.92 | 0.80(0.11–5.89) |
| Allele | Frequency | | | |
| Allele G | 142(84.5%) | 116(82.9%) | | 1 |
| Allele C | 26(15.5%) | 24(17.1%) | 0.81 | 0.89(0.48–1.62) |

## 4. Experimental

### *4.1. Subjects and study design*

We designed a case-control study for GC response to investigate the association of GC resistance and MIF polymorphism and expression in ITP. Our study included 84 GC-sensitive patients with ITP and 70 GC-resistant patients with ITP. All patients were recruited locally from Dalian city, China. Patients from three hospitals were included: The First Affiliated Hospital of Dalian Medical University, The Dalian Municipal Central Hospital and The Dalian 210 Hospital. The critera for GC therapy (success or failure) were based on the American Society of Hematology (Neunert et al. 2011). The patients were treated with GC and classified into two groups: GC-resistant and GC-sensitive. The characteristics of the subjects are shown in Table 1. Informed consent was obtained from all the patients. This study was approved by the Research Ethics Committee of Dalian Medical University. The peripheral leucocytes cell fraction was separated by Lymphoprep density-gradient centrifugation (density 1.077 g/m$^3$; Nucomedpharma. Oslo, Norway).

### *4.2. MIF mRNA expression by Semi-quantitative real-time RT-PCR*

Total RNA was extracted from the PBMCs using a Blood Genome RNA Extraction Kit (TaKaRa, Dalian,China) according to the manufacturer's instructions. cDNA was synthesized using an RT-PCR kit (Stratagene, Cedar Creek, TX) as described previously by Leech et al. (1999). The levels of MIF mRNA were analyzed using semi-quantitative RT-PCR. The primers sequences were as follows; for MIF 5′-CAC AGT GGT GTC CGA GAA GTC AGG-3′(forward) and 5′-GCG TCC CTG GGT GCG ACA GAC GCGA-3′ (reverse), yielding a PCR product of 381 bp; for GAPDH 5′-GTT GCC ATC AAT GAC CCC TTC ATTG-3′ (forward) and 5′-GCT TCA CCA CCT TCT TGA TGT CATC-3′(reverse), yielding a PCR product of 300 bp. PCR was performed in a 25-ml volume with 20 mM Tris HCl (pH 8.4), 50 mM KCl,3 mM MgCl, 400 mM dNTPs, 500 nM each of forward and reverse cDNA primers, 2 ml of cDNA, and 2.5 units of Taq DNA polymerase (Gibco BRL). Cycle conditions were as follows; 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min (Leech et al. 1999). Each sample was analyzed in at least two independent assays with duplicate samples.

### *4.3. Analysis of serum MIF by Enzyme-linked immunosorbent assay*

Quantitative analysis of MIF levels in serum was performed by Enzyme-linked immunosorbent assay according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The range of detection was of 0.156–10 ng/mL and the sensitivity of 0.017 ng/mL. The samples were diluted 10 times before the assay.

### *4.4. Genotyping for the MIF MIF-173G/C polymorphism*

Peripheral blood samples were collected in vacuum tubes containing 5% EDTA. Genomic DNA was extracted using DNA Purification Kit (TaKaRa, Dalian, China) according to the instructions. All subjects were genotyped for MIF-173G/C SNP using Polymerase chain reaction (PCR) Tm-shift genotyping method with fluorescence melting curve analysis on an Roche Light Cycler 480 (Roche Bio Inc, Rotkreuz, Switzerland) (Li et al. 2011). Two allele-specific forward primers with the 3'base of each primer matching one of the allele bases of the SNP were used. The sequences of forward primers were rs755622G: GGCGCGCAGGGGGGCTAGCCGCCAAGTG-GAGAACTGG and rs755622C: TAGCCGCCAAGTGGAGA ACTGC. A common reverse primer sequence was rs755622R: TTCCTCCAGCAAC-CGCCGCT. Cycle conditions were as follows; after initial denaturation at 95 °C for 15 s, PCR was performed for 30 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 34 s. The fluorescence melting curve was analyzed immediately following amplification. For 10% of the samples were repeated for quality control.

### *4.5. Statistical analysis*

All the data were expressed as the mean ± SD. The software SPSS 13.0 was used for the statistics analysis (SPSS Inc., Chicago, IL, USA). The differences between the groups were evaluated using the Chi-square test or Fisher's exact test. $p < 0.05$ was considered as statistically significant.

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