RESEARCH ARTICLE

Disease-specific signature of serum miR-20b and its targets IL-8 and IL-25, in myasthenia gravis patients

Nie Chunjie[1], Nie Huijuan[1], Yin Zhao[1], Wu Jianzhao[2], Zhang Xiaojian[1]

[1] Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, P.R. China
[2] Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Correspondence: N. Chunjie. Department of Pharmacy, The First Affiliated Hospital of Zhengzhou University, No. 1, Jianshe Road, Zhengzhou 450052, China
<niechunjie2010@163.com>

To cite this article: Chunjie N, Huijuan N, Zhao Y, Jianzhao W, Xiaojian Z. Disease-specific signature of serum miR-20b and its targets IL-8 and IL-25, in myasthenia gravis patients. *Eur. Cytokine Netw.* 2015; 26(3): 61-6 doi:10.1684/ecn.2015.0367

ABSTRACT. Myasthenia gravis (MG) is an autoimmune disorder characterized by antibodies directed against components of the neuromuscular junction. Currently, the diagnosis and therapeutic evaluation rely on the serum acetylcholine receptor (AchR) antibody titer, which is not reliable for monitoring. The disruption of the menus had been implicated in many immunological disorders, including MG. A quantitative PCR was used to evaluate the miR-20b level. ELISA was used to determine the levels of IL-8 and IL-25 in serum. Quantitative MG scores (QMGS) were used to examine the clinical manifestations. Here, we report that miR-20b, an immune- and cancer-related miRNA, is decreased in the serum of MG patients and correlates negatively with QMGSs in the pretreatment stage. Furthermore, after treatment with prednisone acetate, levels of miR-20b recover but remain negatively correlated with the QMGS. We also identified that IL-8 and IL-25 are targets of miR-20b via the luciferase reporter system. Both of these are increased in MG and correlate negatively with miR-20b. Furthermore, IL-8 and IL-25 levels are decreased following treatment with prednisone acetate. Our data suggest that miR-20b might be a potential biomarker for MG.

Key words: myasthenia gravis, miRNA, IL-8, IL-25

Myasthenia gravis (MG) is a chronic, autoimmune, neuromuscular disorder characterized by fluctuating muscle weakness and fatigability, worsening with exertion, and alleviating with rest [1]. Patients with MG have been reported to have specific autoantibodies directed against important components of the muscle membrane at the neuromuscular junction, such as the acetylcholine receptor (AChR), muscle-specific tyrosine kinase (MuSK) and lipoprotein-related protein 4 (LRP4) [2-4]. Clinically, MG can be divided to two types, ocular MG and generalized MG [5], based on the location of the affected muscles, or early-onset MG (EOMG), when symptoms appear before 50 years of age, late-onset MG (LOMG), when they appear after 50 years, or very late-onset MG (VLOMG), when symptoms appear after 60 years of age [6]. Experimentally, MG is usually classified or diagnosed according to the different type of autoantibodies present, as described above. However, the serum antibody titer is not consistent with the degree of muscle weakness or the response to therapy [7], so cannot be used as a reliable biomarker for prediction of the disease severity for individual treatment regimens and clinical trials.

Mammalian microRNAs (miRNAs) are small, non-coding, regulatory molecules that regulate gene expression by complementary binding to the 3' untranslated region of their target messenger RNAs thus inhibiting their translation [8]. MiRNAs are important for several cellular functions such as differentiation, apoptosis and proliferation due to their ability to fine-tune gene expression [9]. In the immune system, miRNAs are also critical as they participate in immune cell development, germinal center responses, generation of Ig class-switched plasma cells, and responses to toll-like receptor stimuli [10]. The abnormal alterations of miRNAs had been implicated in several immunological diseases, including MG [11]. Increasing evidence suggests that miRNAs can be detected and quantitatively analyzed in biofluids, including serum, plasma, urine, and saliva [12]. The detection of circulating miRNAs in patient biofluids has been considered to be a novel method of detecting the progression of cardiovascular diseases and malignant growth [13]. It is possible to assay circulating miRNAs and used them as readily accessible blood biomarkers to monitor disease state in MG.

In this study, we investigated the expression profile of miR-20b, a miRNA that is decreased in MG patients as reported previously, in the pathogenesis of MG, and the potential application of miR-20b detection and its direct targets, IL-8 and IL-25 in the diagnosis of MG and evaluation of its treatment.

doi: 10.1684/ecn.2015.0367

Table 1
The demographic characteristics of study subjects

Information	MG patients	Ocular MG	Generalized MG	Health Control
Number of cases	32	18	14	28
Age (years)	37.6 yrs 12.3	35.7 yrs 9.6	39.2 yrs 7.5	38.3 yrs 8.4
Female/male	18/14	10/8	8/6	15/13
Disease duration (months)	15.2 ± 6.8	13.6 ± 4.7	18.0 ± 5.6	N.A.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Ethics Committee of Zhengzhou University and performed in accordance with the Declaration of Helsinki for Human Research. All participants gave written, informed consent for inclusion.

Subjects

All MG patients and the healthy controls that were enrolled in this study were from the first Affiliated Hospital of Zhengzhou University. The criteria for inclusion consisted of: (1) early-onset MG with objective clinical muscle fatigue along with disturbed neuromuscular transmission on repetitive nerve stimulation and/or single-fiber electromyography, (2) the presence of serum anti-acetylcholine receptor antibodies, (3) no thymoma, and (4) no immunosuppressive treatment for at least six months prior to the study. Serum samples were obtained from 32 MG patients (before prednisone acetate treatment, one month and three months after prednisone acetate treatment), and from 28, age-matched, healthy blood donors with no inflammatory diseases. All patients were classified into one of two groups: ocular MG (18 patients) or generalized MG (14 patients). Patient details are summarized in *table 1*. A quantitative myasthenia gravis score (QMGS) was used to evaluate the clinical manifestations of MG [14].

RNA isolation and miRNA expression analysis

Blood samples were collected in sterilized EP tubes and centrifuged at room temperature, at 1300 × g for 10 min to collect the serum. The samples were then aliquoted and stored at -80 °C until used. RNA isolation and CDNA preparation were performed using a miRcute miRNA Isolation Kit (Tiangen, Beijing, China), according to the manufacturer's instructions. Real-time PCR cycle conditions included the following steps: miR-20b, denaturation at 95 °C for 3 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. To normalize the expression levels of target miRNAs, U6 was used as reference. The primers for has-miR-20b detection were purchased from Tiangen Biotech (CD201-0091). The U6 snRNA primers were used as follows: sense: 5' ATTGGAACGATACAGAGAAGATT 3'; U6 snRNA antisense: 5'GGAACGCTTCACGAATTTG 3'.

Dual luciferase assay

The wild type or mutated 3'UTR sequences of human IL-8 and IL-25 were cloned and inserted into the psiCHECK-2 vector. HEK293 cells were cultured in a 96-well plate with a density of 6000 per well. Twenty four hours later, the cells were co-transfected with 0.6 μl lipofectamine 2000 (Invitrogen, NY, USA), 0.05 μg psiCHECK2-IL-8 or psiCHECK2-IL-25, and 0.15 μg has-miR-20b mimics per well. Forty eight hours after transfection, cells were harvest for dual luciferase activity assay using the dual-luciferase reporter assay kit (Beyotime Biotechnology, Shanghai, China).

Enzyme-linked immunosorbent assay (ELISA)

Serum IL-8 and IL-25 levels were measured with ELISA kits (Jianglai Lab, Shanghai, China) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± SD. Differences between two groups were determined using a two-tailed Student's t test, and multiple comparisons were determined by ANOVA. Serum IL-8 and IL-25 levels, miR-20b levels, and the OMGS were correlated by parametric Pearson correlation analysis. p values <0.05 were considered statistically significant.

RESULTS

Serum miR-20b levels in MG patients

We first compared serum miR-20b levels in MG patients and healthy controls using real-time quantitative RT-PCR. We found that the levels of miR-20b were dramatically decreased in the MG patients as reported previously [15]. The miR-20b expression in generalized MG patients was much lower than that found in ocular MG patients (*figure 1*). We then evaluated the miR-20b levels following treatment with glucocorticoid. We found that one month after treatment with prednisone acetate, the serum levels of miR-20b increased when compared to the untreated samples. However, it was only after three months of treatment that the miR-20b levels returned to normal (*figure 2*). These data suggested that miR-20b is decreased in MG patients but gradually recovers following glucocorticoid treatment.

Correlation analysis between the QMGS and miR-20b

To test whether the changes in serum miR-20b levels in MG correlate with the clinical manifestations, we performed a correlation analysis with QMGS and serum miR-20b for the 32 MG patients. We found the serum miR-20b correlates negatively with the QMGS. Importantly, the R squared in the pretreatment and three-month treatment stages are much higher than in the one month treatment

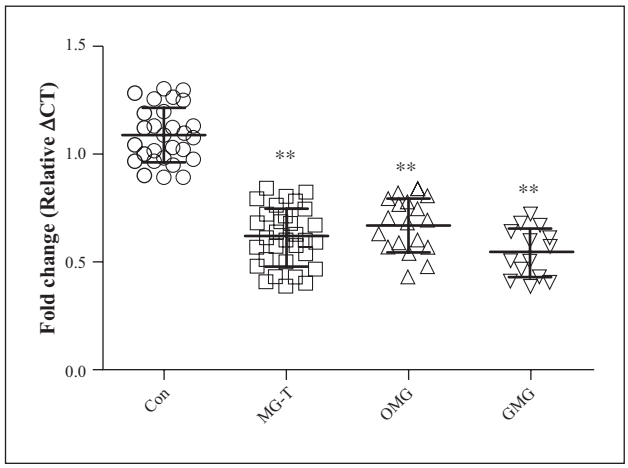

Figure 1

Serum miR-20b levels are decreased in MG patients. The serum of MG patients was collected as described above. The levels of miR-20b were measured according to the manufacturer's instructions. Con, health control; MG-T, all the MG samples; OMG, ocular MG samples; GMG, generalized MG samples. ** $p<0.01$, compared with Con.

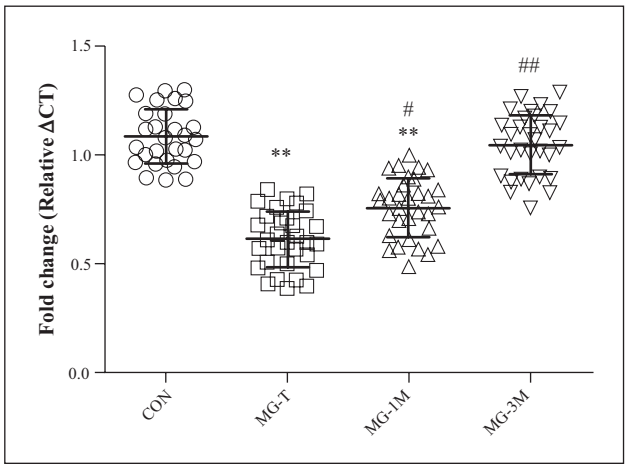

Figure 2

Serum miR-20b levels are restored after glucocorticoid treatment. The serum of MG patients was collected as described above. The levels of miR-20b were measured according to the manufacturer's instructions. Con, health control; MG-T, all the MG samples; MG-1M, 1-month treatment MG samples; MG-3M, 3-month treatment MG samples. ** $p<0.01$, compared with Con, #$p<0.05$, ##$p<0.01$, compared with MG-T.

stage (*figure 3*). These data suggested that serum miR-20b might be used as a parameter for diagnostic and therapeutic effect evaluation.

IL-8 and IL-25 are the direct targets of miR-20b

It is known that miRNAs regulate the expression of their targets by binding directly to the 3'UTR of target mRNA and inhibit translation. We then used the online prediction tools TargetScan 6.2 and miRnada to predict the potential gene targets of miR-20b. We found that both interleukin-8 and interleukin-25 were included in the outputs of the two independent prediction tools. To verify the bioinfomatics prediction, we used the luciferase reporter system. We cloned the 3'UTR of human IL-8 and IL-25 fragments into the psiCheck2 vector, and performed site-mutagenesis to generate a mutant 3'UTR that could not be recognized by miR-20b. We transfected both the wild-type and the mutated ones into the HEK293 cells and co-transfected them with has-miR-20b mimics or scrambled control. We found that has-miR-20b mimics significantly decreased the luciferase activity of wild-type IL-8 and IL-25 reporters, but not mutant ones (*figure 4*). Thus, IL-8 and IL-25 are direct targets of miR-20b.

IL-8 and IL-25 are increased in the serum of MG patients

As the level of miR-20b, a natural inhibitor of IL-8 and IL-25, is decreased in MG, we then queried how IL-8 and IL-25 are changed in MG patients. Using ELISA, we found that both IL-8 and IL-25 are increased equally in the serum of OMG and GMG patients (*figure 5*). Meanwhile, miR-20b correlated negatively with IL-8 and IL-25 (*figure 6*), further indicating the direct regulation of IL-8 and IL-25 by miR-20b.

IL-8 and IL-25 are decreased in the serum of MG patients following prednisone acetate treatment

We also evaluated the levels of IL-8 and IL-25 in the serum of MG patients following prednisone acetate treatment. We

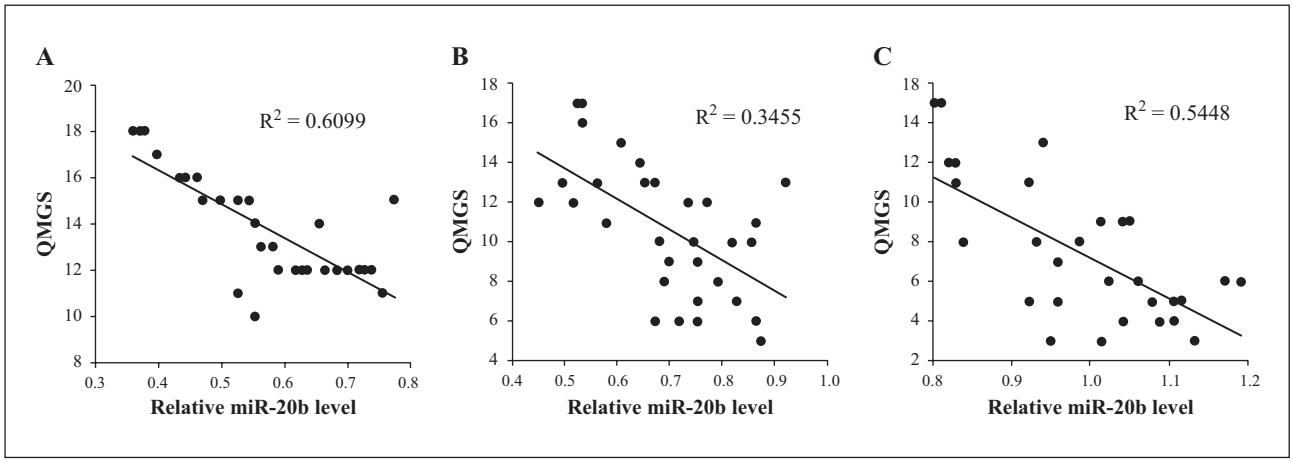

Figure 3

Negative correlation of serum miR-20b with QMGS in three stages. The QMGS evaluations were performed as above. The correlation analyses were performed using SPSS. **A)** MG patients before treatment. **B)** MG patients after 1 month of treatment. **C)** MG patients after 3 months of treatment.

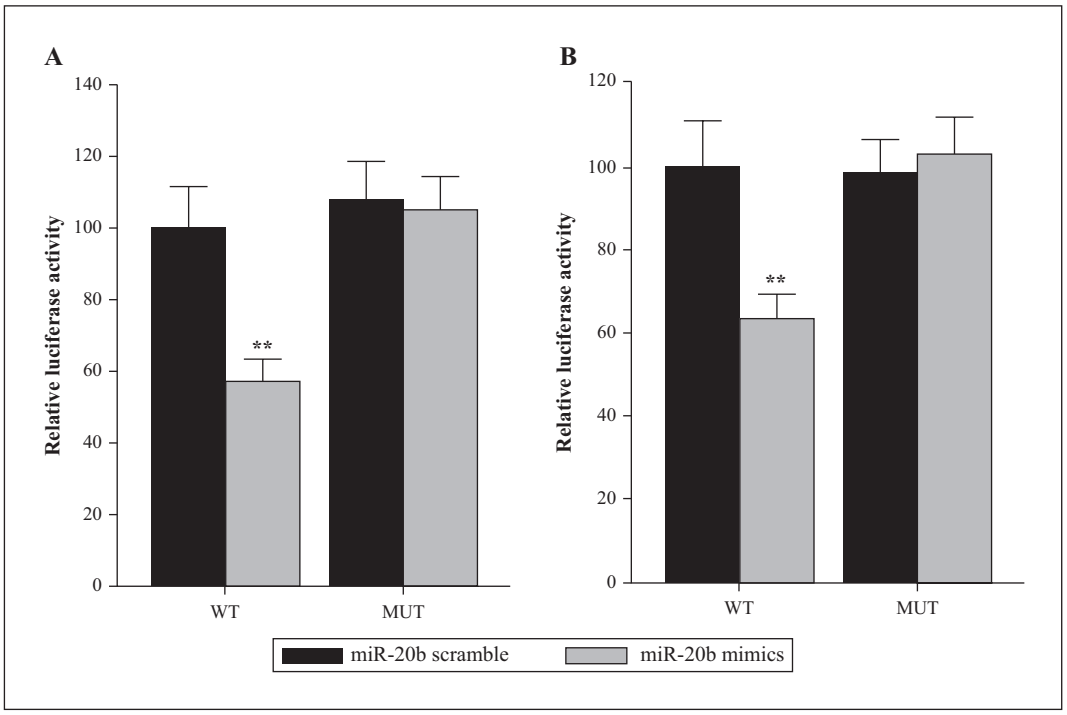

Figure 4

miR-20b directly regulates IL-8 and IL-25. The wild type and binding site mutant fragment of 3'UTR in IL-8 or IL-25 mRNA were co-transfected with miR-20b scrambled control and miR-20b mimics. Luciferase was measured using commercial kits. **A)** IL-8 contructs. **B)** IL-25 constructs. ** P<0.01, compared with wild-type fragment plus scrambled control.

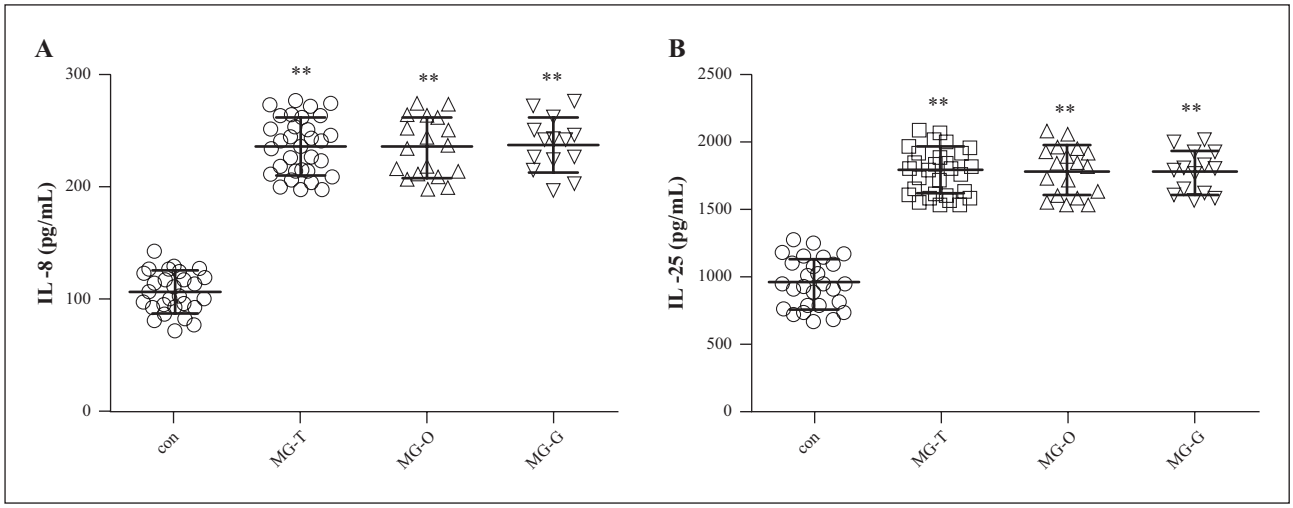

Figure 5

Serum IL-8 and IL-25 levels are increased in MG patients. The serum of MG patients was collected as described above. The levels of IL-8 and IL-25 were measured by ELISA. Con, health control; MG-T, all the MG samples; OMG, ocular MG samples; GMG, generalized MG samples. ** p<0.01, compared with Con.

found that after one month of treatment with the glucocorticoid, the serum levels of IL-8 and IL-25 had fallen when compared to the untreated samples. However, IL-8 and IL-25 levels did not return to normal levels until after three months of treatment (*figure 7*). These data are consistent with the above results and suggest that the serum levels of IL-8 and IL-25 might also be used for the evaluation of therapeutic effects.

DISCUSSION

In this study, we have reported that decreased serum miR-20b in MG patients correlated negatively with the QMGS and targets, IL-8 and IL-25, which are upregulated in MG. We also found that the level of miR-20b is restored to normal upon glucocorticoid treatment and could be useful as a therapeutic evaluation biomarker.

miRNAs are small, non-coding RNAs that are found in many diseases. Several miRNAs have been suggested to play important roles in the pathogenesis of MG [15]. As such, a correlation has been reported between decreased levels of miR-320a and increased proinflammatory cytokines such as IL-2 and IL-17 in peripheral mononuclear cells (PBMCs) in MG [16]. In the serum of MG patients, the level of miR-27a is reduced [17], which may reflect increased NK cell activation [18]. Meanwhile, both miR-150 and miR-21 are increased in MG serum, and

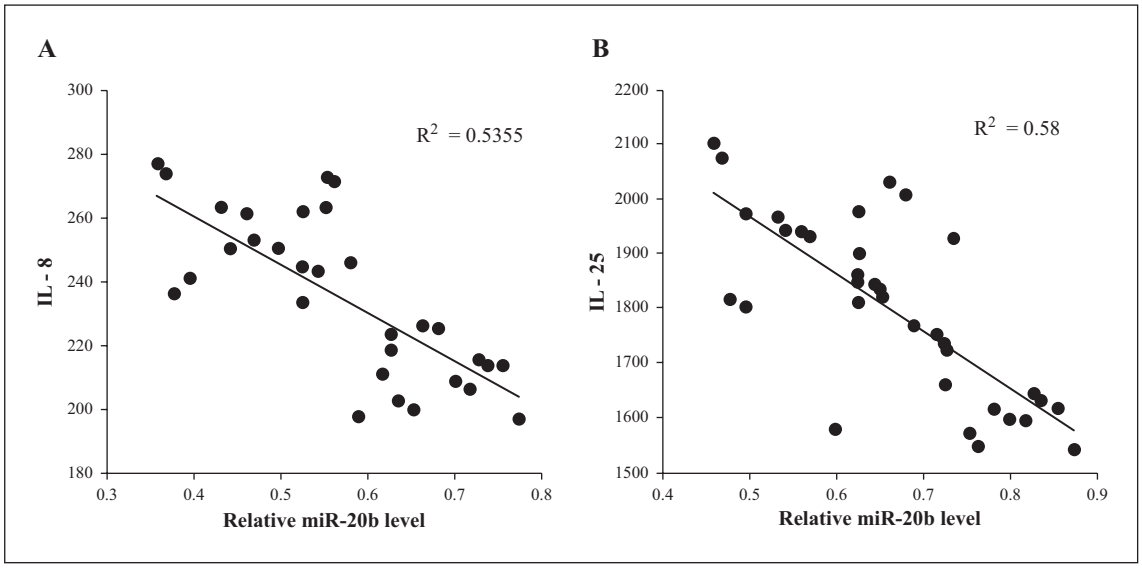

Figure 6

Negative correlation of serum IL-8 and IL-25 with QMGS. The QMGS evaluations were performed as above. The correlation analyses were performed using SPSS. **A)** correlation of IL-8 with QMGS, **B)** correlation of IL-25 with QMGS.

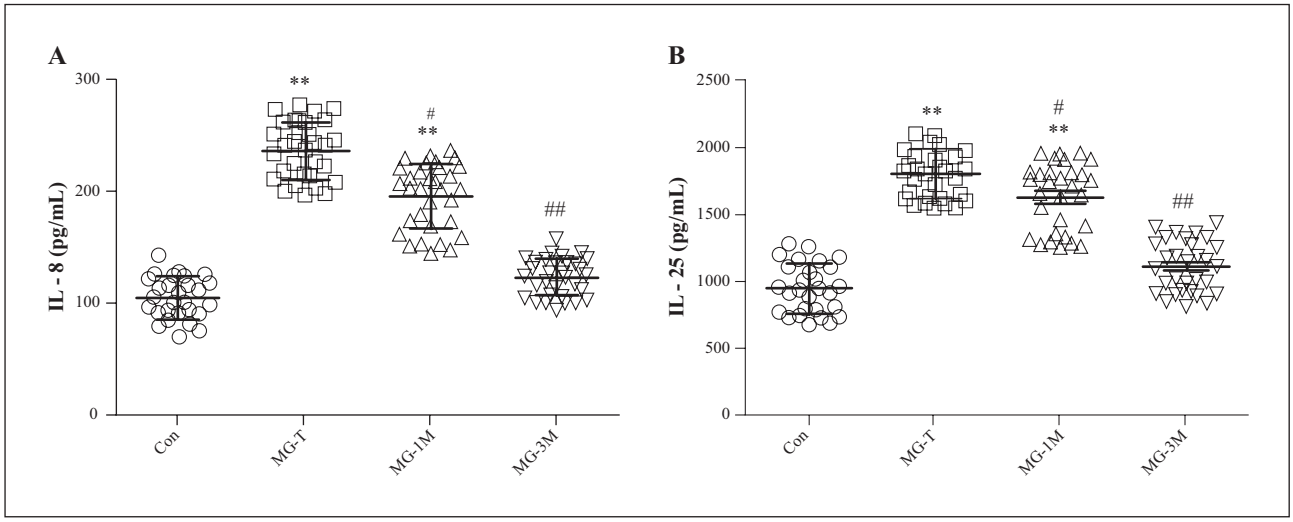

Figure 7

Serum IL-8 and IL-25 levels are decreased in MG patients following prednisone acetate treatment. The serum of MG patients was collected as described above. The levels of IL-8 and IL-25 were measured by ELISA. Con, health control; MG-T, all the MG samples; MG-1M, 1-month treatment MG samples; MG-3M, 3-month treatment MG samples. ** $p<0.01$, compared with Con, #$p<0.05$, ##$p<0.01$, compared with MG-T.

significantly decreased after thymectomy or under stable immunosuppressive drug treatment over 6 months, which correlates well with accompanying clinical improvement [17, 19]. Here, we have shown that miR-20b is down-regulated in the serum of MG patients but increases after the treatment. Moreover, the level of miR-20b correlates strongly with the QMGS at different stages.

miR-20b belongs to the miR-17 family, and has been implicated in many diseases, including many cancers and immunological disorders. For example, miR-17, 20a, and 20b are down-regulated in cisplatin-resistant A549/DDP cells compared with A549 cells. Inhibition of miR-17, 20a, and 20b increased cisplatin-resistance and migration of A549 cells. Over-expression of miR-17, 20a, and 20b decreased cisplatin-resistance and migration of A549/DDP cells. In addition, miR-17, 20a, and 20b blunted the TGFβ signalling pathway by directly inhibiting its important component TGFβR2 [20]. An inhibitor of miR-20b dramatically suppressed HCC1806 breast cancer cell proliferation and migration resulting in a G0/G1 and S phase arrest in the cell cycle by targeting tumor suppressors PTEN and BRCA1, which further indicated the key role of miR-20b in the development of this disease [21]. The decrease in miR-20b thatis observed in MSCs compared with hESs would result in the overexpression of one of its targets, the transcription regulator EPAS1, which allows expression of MSC genes contributing to determination of the MSC phenotype [22]. Overexpression of miR-20b led to decreased Th17 cells and reduced severity of experimental autoimmune encephalomyelitis (EAE). Both RAR-related orphan receptor γt and STAT3 are potential targets of miR-20b in EAE [23]. In the current study, we also verified IL-8 and IL-25 as targets of miR-20b, which are both increased in MG.

Overall, our results are the first to show that miR-20b expression is downregulated in MG, and that it is associated with severity, and the therapeutic effects of one type

of immunological treatment of patients with MG. miR-20b was able to down-regulate the production of the proinflammatory cytokines IL-8 and IL-25.

Disclosure. *Financial support: this study was supported by a grant from the Youth Foundation of The First Affiliated Hospital of Zhengzhou University [YFFAHZZ2013020116] to Nie Chunjie. Conflict of interest: none.*

REFERENCES

1. Jayam Trouth A, Dabi A, Solieman N, Kurukumbi M, Kalyanam J. Myasthenia gravis: a review. *Autoimmune Dis* 2012; 2012: 874680.
2. Vincent A, Palace J, Hilton-Jones D. Myasthenia gravis. *Lancet* 2001; 357: 2122-8.
3. Drachman DB. Myasthenia gravis. *New Eng J Med* 1994; 330: 1797-810.
4. Querol L, Illa I. Myasthenia gravis and the neuromuscular junction. *Cur Opin Neurol* 2013; 26: 459-65.
5. Monsul NT, Patwa HS, Knorr AM, Lesser RL, Goldstein JM. The effect of prednisone on the progression from ocular to generalized myasthenia gravis. *J Neurol Sci* 2004; 217: 131-3.
6. Berrih-Aknin S, Le Panse R. Myasthenia gravis: a comprehensive review of immune dysregulation and etiological mechanisms. *J Autoim* 2014; 52: 90-100.
7. Kaminski HJ, Kusner LL, Wolfe GI, *et al*. Biomarker development for myasthenia gravis. *An N Y Ac Sci* 2012; 1275: 101-6.
8. Zampetaki A, Willeit P, Drozdov I, Kiechl S, Mayr M. Profiling of circulating microRNAs: from single biomarkers to re-wired networks. *Cardiovasc Res* 2012; 93: 555-62.
9. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? *Trends Cell Biol* 2007; 17: 118-26.
10. Pauley KM, Cha S, Chan EK. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun* 2009; 32: 189-94.
11. Le Panse R, Berrih-Aknin S. Autoimmune myasthenia gravis: autoantibody mechanisms and new developments on immune regulation. *Cur Opin Neurol* 2013; 26: 569-76.
12. Etheridge A, Lee I, Hood L, Galas D, Wang K. Extracellular microRNA: a new source of biomarkers. *Mutat Res* 2011; 717: 85-90.
13. Creemers EE, Tijsen AJ, Pinto YM. Circulating microRNAs: novel biomarkers and extracellular communicators in cardiovascular disease? *Circ Res* 2012; 110: 483-95.
14. Barth D, Nabavi Nouri M, Ng E, Nwe P, Bril V. Comparison of IVIg and PLEX in patients with myasthenia gravis. *Neurology* 2011; 76: 2017-23.
15. Nogales-Gadea G, Ramos-Fransi A, Suarez-Calvet X, Navas M, Rojas-Garcia R, Mosquera JL, *et al*. Analysis of serum miRNA profiles of myasthenia gravis patients. *PloS One* 2014; 9: e91927.
16. Cheng Z, Qiu S, Jiang L, *et al*. MiR-320a is downregulated in patients with myasthenia gravis and modulates inflammatory cytokines production by targeting mitogen-activated protein kinase 1. *J Clin Immunol* 2013; 33: 567-76.
17. Punga T, Le Panse R, Andersson M, Truffault F, Berrih-Aknin S, Punga AR. Circulating miRNAs in myasthenia gravis: miR-150-5p as a new potential biomarker. *Ann Clin Transl Neurol* 2014; 1: 49-58.
18. Kim TD, Lee SU, Yun S, *et al*. Human microRNA-27a* targets Prf1 and GzmB expression to regulate NK-cell cytotoxicity. *Blood* 2011; 118: 5476-86.
19. Punga AR, Andersson M, Alimohammadi M, Punga T. Disease specific signature of circulating miR-150-5p and miR-21-5p in myasthenia gravis patients. *J Neurol Sci* 2015 Jun 14.
20. Jiang Z, Yin J, Fu W, *et al*. MiRNA 17 family regulates cisplatin-resistant and metastasis by targeting TGFbetaR2 in NSCLC. *PloS One* 2014; 9: e94639.
21. Li D, Ilnytskyy Y, Kovalchuk A, *et al*. Crucial role for early growth response-1 in the transcriptional regulation of miR-20b in breast cancer. *Oncotarget* 2013; 4: 1373-87.
22. Giraud-Triboult K, Rochon-Beaucourt C, Nissan X, Champon B, Aubert S, Pietu G. Combined mRNA and microRNA profiling reveals that miR-148a and miR-20b control human mesenchymal stem cell phenotype via EPAS1. *Physiol Genomics* 2011; 43: 77-86.
23. Zhu E, Wang X, Zheng B, *et al*. miR-20b suppresses Th17 differentiation and the pathogenesis of experimental autoimmune encephalomyelitis by targeting RORgammat and STAT3. *J Immunol* 2014; 192: 5599-609.