High serum level of haptoglobin is associated with the response of 12 weeks methotrexate therapy in recent-onset rheumatoid arthritis patients

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Abstract
Background: We previously found, using microarray, haptoglobin (HP) expression signal was 5.1-fold increased in peripheral blood mononuclear cells (PBMCs) from methotrexate (MTX)-resistant rheumatoid arthritis (RA) patients.
Objectives: To investigate whether serum levels of HP are associated with the response of 12 weeks MTX therapy in recent-onset RA patients.
Methods: Sixty-nine active RA patients with recent onset (< 24 months) were treated with MTX. Clinical variables, levels of HP messenger RNA (mRNA) in PBMCs and HP serum levels were tested at week 0 and week 12.
Results: After 12 weeks of MTX treatment, 34.7% of RA patients were categorized as responders according to European League Against Rheumatism (EULAR) response criteria (Week 12 Disease Activity Score of 28 joints [DAS-28] ≤ 3.2 and decrease > 1.2) and all others (65.2%) were defined as non-responders. The baseline HP mRNA in PBMCs from non-responders is significantly higher than those in responders (P < 0.05). Similar to mRNA expression, non-responders showed significantly elevated serum HP levels at baseline (369.9 ± 159.8 mg/dL) compared to those in responders (255.3 ± 143.9 mg/dL) (P = 0.01). Serum HP levels were decreased significantly from 255.3 ± 143.9 mg/dL at baseline to 186.4 ± 108.5 mg/dL at week 12 (P = 0.04) in responders, but remained at high levels in non-responders.
Conclusions: High serum levels of HP at baseline are associated with inadequate response of 12 weeks MTX treatment in recent-onset RA patients. Further replication studies in larger samples are needed to validate HP as a potential predictive biomarker for response to MTX therapy in RA.
Key words: haptoglobin, methotrexate, rheumatoid arthritis.

INTRODUCTION

Methotrexate (MTX) is currently the most widely used drug for rheumatoid arthritis (RA) treatment, due to its high efficacy for disease modification both in monotherapy and in combination therapy with other disease-modifying anti-rheumatic drugs (DMARDs). Given 30–40% of the patients could experience an adequate response to MTX alone,¹,² it might not be necessary to start intensive combination therapy for all newly diagnosed RA patients.³,⁴ Thus, it is important to find out predictive biomarkers to identify patients who might be most likely to respond or not respond to MTX.
monotherapy. Several factors have been tested for the prediction of response to MTX in RA patients, including demographic, clinical, immunologic and genetic factors; however, none of these have sufficient predictive power to be helpful in making therapeutic decisions in daily clinical practice.

In the past, using microarray genome-wide analysis of gene expression profiles of peripheral blood mononuclear cells (PBMCs), we have discovered a small set of discriminative transcripts between MTX-responsive and MTX-resistant RA patients. In this set, haptoglobin (HP, OMIM:140100) expression signal was 5.1-fold increased in MTX-resistant RA patients (Tan W, Wang F, Guo M, Zhang M. unpubl. data).

The primary physiological function of HP is to bind free hemoglobin to prevent oxidative damages to various organs. Recently, HP has been suggested to participate actively in several processes of innate and adaptive immune responses by activation of innate and adaptive immune cells at local and systemic levels. HP could specifically interact with both resting and activated CD4+ and CD8 + T cells and modulate the Th1/Th2 balance by promoting a dominant Th1 cellular response. HP can bind to CD22 on B cells, involved in B cell activation and survival. Most recently, HP has been suggested as an innate immune ligand to activate myeloid differentiation primary response gene 88 (MyD88) in CD11c+ dendritic cells (DCs), contributing to the inflammatory response of DCs and innate immune activation.

Collectively, the tight correlations between HP and immune cells implies that HP might play an important role in RA pathogenesis.

HP is synthesized mainly in the liver, with very low expression in other tissues. During acute-phase reaction processes such as inflammation and tissue injury, HP expression is increased by two- to 10-fold; thus, it also functions as an acute-phase reactant protein. High serum levels of HP have been found in malignancies, atherosclerosis, type 1 diabetes, inflammatory bowel diseases and several autoimmune diseases, including systemic lupus erythematosus, RA and juvenile idiopathic arthritis (JIA). Significant elevation of HP levels in RA synovial fluid and arthritic tissues has been correlated with disease activity. More importantly, it was recently reported that RA patients with high levels of HP in serum were more likely to have poor disease control. These observations and our previous microarray data prompted us to investigate whether serum HP levels are associated with the response of MTX treatment in RA patients.

PATIENTS AND METHODS

Patients

A total of 69 newly diagnosed RA patients were included in this 12-week follow-up study. The criteria for patient eligibility were: (i) fulfillment of the revised 1987 American Rheumatism Association criteria for RA; (ii) naïve to DMARDs or biologics treatment; (iii) recent-onset RA with active disease (defined as < 2 years from onset of symptoms to first presentation to the rheumatologist and with a Disease Activity Score of 28 joints [DAS-28] > 3.2). All patients began treatment with a regimen of oral MTX at a dosage of 10 mg weekly, with the dosage increasing to 20 mg weekly after 4–6 weeks. Non-steroidal anti-inflammatory drugs were permitted but glucocorticoids were not allowed in this study.

Clinical variables associated with disease activity included DAS-28, erythrocyte sedimentation rate (ESR), serum C-reactive protein (CRP) level, patient’s self-assessment of pain (0–100 mm visual analogue scale [VAS]), Health Assessment Questionnaire (HAQ), Short Form – 36 (SF-36) 10-item Physical Functioning Scale and duration of morning stiffness score were recorded at week 0 (the time of MTX therapy), weeks 4 and 12 of treatment. After 12 weeks of treatment, the patients were categorized as responders (week 12 DAS-28 ≤ 3.2 and decrease > 1.2) and non-responders (all others) using the European League Against Rheumatism (EULAR) response criteria. This study was approved by the First Affiliated Hospital of Nanjing Medical University Committee on Human Research and conformed to the Helsinki Declaration of the World Medical Association. All subjects in this study provided written informed consent.

Measurement of HP in PBMCs and serum

Blood samples were extracted at week 0 (RNA and serum samples) and week 12 (serum samples only) of MTX treatment from all patients and sex- and age-matched healthy controls (HC). HP messenger RNA (mRNA) expression levels in PBMCs from 69 baseline RA patients and 36 HCs were measured by ABI TaqMan assay (HP: NM_005143.3, ABI assay ID Hs00978377_m1; Applied Biosystems, Beijing, China). Relative HP expression in PBMCs was normalized by the expression level of ribosomal protein (RPLPO, ABI assay ID Hs00978377_m1; Applied Biosystems). The relative expression (RE) of HP transcript was calculated based on the fold expression compared to the lowest expression level. Experiments were performed in duplicate for
each sample in 384-well plates using the Applied Biosystems 7900 real-time polymerase chain reaction (PCR) system. Data were analyzed using SDS software (Applied Biosystems).

Concentrations of levels of HP in serum of RA patients and HC at baseline and week 12 were quantified using a commercial, enzyme-linked immunosorbent assay (ELISA) kit from ALPCO Diagnostics (Salem, NH, USA). ELISA was performed according to the manufacturer's instructions.

Statistical analysis
All statistical analyses were performed using software package Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). Comparisons between groups were performed by Mann–Whitney U-test. Correlation between HP and laboratory indices and clinical data was analyzed by a Spearman rank test. A multivariate logistic regression model was used to find the independent predictive value of the biomarker on response of 12 weeks MTX treatment. The area under the curve (AUC) and best cut-off point was calculated employing receiver operating characteristic (ROC) analysis. Results were considered significant when \( P < 0.05 \).

RESULTS
Demographics of study patients
Tables 1 and 2 show the demographic and clinical characteristics of these 69 RA patients at study baseline. A total of 92.4% of patients were female, 71.1% were immunoglobulin M (IgM) rheumatoid factor (RF) positive and 52.3% were anticyclic citrullinated peptide antibody (anti-CCP) positive. Before treatment, all variables (except VAS) were similar for responders and non-responders. Mean baseline DAS-28 was 5.0 ± 0.9 for all patients and 5.2 ± 0.7 for responders and 4.8 ± 0.9 for non-responders, indicating these patients had high disease activity.

Responses to MTX treatment
The clinical response to MTX was evaluated using EULAR criteria, and disease activity was assessed using DAS-28 based on ESR. After 12 weeks of MTX treatment, 34.7% (\( n = 24 \)) of all 69 evaluated patients reached good response status according EULAR criteria and all others with inadequate response (65.2%, \( n = 45 \)) were classified as non-responders. Under treatment, the mean DAS-28 at week 12 was decreased from 5.2 ± 0.7 to 3.9 ± 1.1 in responders (\( P < 0.05 \)); however, non-responders retained high DAS-28 scores (4.6 ± 1.2 at week 12 vs. 4.8 ± 0.9 at baseline). Responders’ swollen joint count, VAS, ESR and CRP levels were significantly improved at week 12 (\( P < 0.05 \)), whereas non-responders showed no significant changes in these variables, except ESR and CRP levels (Tables 2).

HP transcript expression in baseline PBMCs from responders and non-responders
We first validated our previous microarray data using real-time PCR. Pre-treatment PBMCs from 24 responders, 45 non-responders and 36 HCs were used to assay the HP transcript levels. At baseline, mean HP expression was about two-fold higher in PBMCs from RA patients than those in HCs (\( P = 0.004 \)). Similar to the HP transcript expression in microarray data, non-responders displayed notably increased levels of baseline HP expression in PBMCs compared to responders (\( P = 0.002 \)) (Fig. 1a).

Serum HP levels at baseline and week 12 in responders and non-responders
Serum levels were measured both at baseline and at the 12th week of MTX treatment. Similar to mRNA expression in PBMCs, baseline serum HP levels were significantly increased in RA patients compared to HCs (\( P < 0.0001 \)). Patients who showed inadequate response to 12 weeks of MTX therapy conferred significantly elevated HP levels at baseline compared to responders (369.9 ± 159.8 mg/dL vs. 255.3 ± 143.9 mg/dL, responders vs. non-responders, \( P = 0.01 \)) (Fig. 1b). Notably, accompanied by the suppression of disease activity by 12 weeks of MTX treatment, serum HP levels decreased significantly from 255.3 ± 143.9 mg/dL at baseline to 186.4 ± 108.5 mg/dL at week 12 (\( P = 0.04 \)) in responders; however, the non-responders retained high levels of HP at week 12 (369.9 ± 159.8 mg/dL at

| Table 1: Demographic and clinical characteristics between methotrexate responders and non-responders |
|---------------------------------------------------------------|
| **Parameter** | **Responders** | **Non-responders** |
| Age, years | 42.3 ± 9.4 | 43.6 ± 11.6 |
| Female, % | 89.1 | 95.5 |
| Duration of symptoms, weeks | 22.7 ± 9.6 | 23.5 ± 7.9 |
| Rheumatoid factor positive, % | 68.6 | 74.3 |
| Anticyclic citrullinated peptide antibody positive, % | 47.1 | 56.6 |

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Table 2  Clinical characteristics at study baseline and after 12 weeks of methotrexate (MTX) treatment

| Parameter (mean±SD) | Responders (n = 24) | Non-responders (n = 45) | Responders (n = 24) | Non-responders (n = 45) |
|--------------------|----------------------|-------------------------|----------------------|-------------------------|
| DAS-28             | 5.2 ± 0.7            | 4.8 ± 0.9               | 3.9 ± 1.1*           | 4.6 ± 1.2               |
| SWJ                | 11.6 ± 4.3           | 13.2 ± 3.6              | 8.6 ± 3.3*           | 10.4 ± 3.3              |
| VAS                | 75.1 ± 22.4**        | 65.8 ± 19.4             | 39.1 ± 15.2*         | 63.3 ± 17.9             |
| HAQ                | 1.8 ± 0.4            | 1.8 ± 0.6               | 1.2 ± 0.6            | 1.5 ± 0.7               |
| ESR (mm/h)         | 44.2 ± 11.7          | 50.3 ± 9.8              | 19.1 ± 17.6*         | 28.6 ± 21.9*            |
| CRP (mg/L)         | 28.1 ± 11.1          | 31.3 ± 12.2             | 7.6 ± 2.3*           | 14.3 ± 18.1*            |

DAS-28, Disease Activity Score of 28 joints; SWJ, swollen joint count; VAS, visual analogue scale (patient’s self-assessment of pain, 0–100 mm); HAQ, Health Assessment Questionnaire; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

Responders were defined as week 12 DAS-28 ≤ 3.2 and decrease > 1.2; all others who had inadequate response to 12 weeks MTX therapy were defined as non-responders.

*P < 0.05, 12 weeks versus baseline.

**P < 0.05, responders versus non-responders at baseline.

baseline vs. 309.6 ± 183.9 mg/dL at week 12; P = 0.71) (Fig. 1b,c). The area under the ROC curve (AUC) between non-responders and responders was 0.76 (Fig. 2). When an optimal cut-off value of 344.1 mg/dL at baseline for non-responders was applied, the diagnostic sensitivity and specificity for responders were 74.4% and 70.5%, respectively.

Correlation of baseline serum HP levels with demographic, clinical and laboratory features of RA

We further analyzed the correlation of baseline serum HP levels with demographic, clinical and laboratory features of RA, including age, disease duration, RF, anti-CCP, DAS-28, VAS, HAQ, CRP and ESR levels. We found a modest but significant correlation between the baseline serum HP levels and ESR (r = 0.38; P < 0.0001), CRP (r = 0.38; P < 0.0001) and DAS-28 (r = 0.15; P < 0.008) (Fig. 3a–c). Since these variables correlated with baseline HP levels, we performed a multivariate logistic regression to determine whether the association between response to the MTX and high levels of HP might have been biased by baseline DAS-28, ESR, CRP and other clinical variables. The results in Table 3 show that baseline serum HP levels independently and significantly correlated with response to MTX in a multivariate model (P = 0.02, OR = 1.61 [1.14–2.73]). We also explored whether treatment response of hydroxychloroquine, one of the other DMARDs, is also related to serum HP levels; however, we failed to confirm an association in a primary study of 36 RA patient and 12 weeks follow-up observation (data not shown).
score, CRP or ESR, the well-known predictors of RA disease activity. When an optimal cut-off value of 344.1 mg/dL was applied, the baseline HP levels might potentially guide choice of therapy by allowing intensive therapy to be avoided in responders or enable early alternative treatment to guide non-responders away from unnecessary MTX exposure. Therefore, it might be wise to take baseline serum levels of HP into consideration when making a decision for RA therapy.

HP is primarily synthesized in the liver and is essential to re-establish systemic homeostasis in response to changes in the extracellular milieu. As a member of the acute phase proteins, serum concentrations of HP could be increased several-fold in the occurrence of local or systemic inflammation or tissue injury. Thus, it is not surprising that the circulating protein and mRNA level of HP is elevated in RA and correlates with disease activity in the present study and previous reports. However, to our knowledge, there have been no reports indicating the association between serum HP levels and response to MTX therapy in RA.

As a folate antagonist, MTX exerts its anti-inflammatory and immunosuppressing action by increasing adenosine release and inhibiting enzymes of folate metabolism, resulting in ineffective nucleic acid synthesis and DNA production. RA patients treated with MTX could normalize up-regulated folate pathway genes, highlighting the essential role of the folate pathway in the action of MTX. Interestingly, a recently study indicated high folate status was associated with high HP protein expression before and following intervention with folic acid, implying that HP might be involved in the folate metabolic pathway. Thus, one of the plausible mechanisms by which HP levels may be associated with response to MTX is through influencing folate metabolism in RA.

Figure 2. Receiver operating curve (ROC) for baseline serum haptoglobin (HP) levels in responders. AUC, area under the curve.

Figure 1. The expression levels of haptoglobin (HP) messenger RNA (mRNA) and secreted protein from non-responders, responders and health controls (HCs). (a) Relative expression of HP in peripheral blood mononuclear cells (PBMCs) at baseline from non-responders (n = 42), responders (n = 27) and HC (n = 36). The mRNA expression results represent the mean ± SD of two independent experiments performed in triplicate. (b,c) Serum levels of HP at baseline (b) and after 12 weeks of methotrexate (MTX) treatment (c). Data are means of triplicate experiments ± SD.
The secretion of HP could be strongly induced by pro-inflammatory cytokines such as interleukin (IL)-6, IL-1 and tumor necrosis factor (TNF)-α. The other possible explanation for our findings is that HP expression is regulated by pro-inflammatory cytokines; in this regard, it seems feasible that high HP levels may be a consequence of activation of multiple inflammatory pathways in RA. In addition, one of the main functions of HP is to bind free hemoglobin (Hb) and then to prevent damage caused by reactive oxygen species generated by free Hb. Oxidative stress has been suggested as an important intracellular signaling molecule that amplifies synovial inflammation and degrades cartilage in RA, therefore, significantly increased HP levels implied the burden of prolonged oxidative stress and tissue repair in RA patients, which might be another explanation for the association between elevated serum HP levels and poor disease activity control by MTX.

During the inflammatory process, elevated HP, in turn, displayed significant anti-inflammatory properties in reducing tissue damage. It has been shown that HP could suppress the secretion of TNF-α, IL-10 and IL-12p70 in macrophages upon lipopolysaccharide stimulation. HP possesses the ability to decrease the biosynthesis of prostaglandins via inhibition of cyclooxygenase and lipoxygenase, suggesting its role in the body’s endogenous defense system against inflammation. Furthermore, the model of experimental autoimmune encephalomyelitis (EAE) showed that Hp−/− mice suffered from a more severe disease and increased production of the pro-inflammatory cytokines interferon (IFN)-γ, IL-17A and IL-6 in the central nervous system, supporting the contribution of HP to the development of autoimmune inflammation. Taken together, it can be hypothesized that in response to

Table 4 Radiological progression according to the Van der Heijde modified Sharp score at 1 year

|                         | Responders (n = 16) | Non-responders (n = 11) | Responders (n = 16) | Non-responders (n = 11) | P  |
|-------------------------|---------------------|-------------------------|---------------------|-------------------------|----|
| Baseline                |                     |                         |                     |                         |    |
| Total score             | 4.57 ± 7.3          | 5.48 ± 9.4              | 7.8 ± 9.1           | 10.4 ± 14.1             | 0.12|
| Erosion score           | 1.79 ± 3.8          | 1.62 ± 4.9              | 2.87 ± 4.9          | 3.0 ± 5.6               | 0.08|
| Joint-space narrowing   | 2.8 ± 5.4           | 3.1 ± 6.7               | 5.1 ± 7.8           | 6.2 ± 8.8               | 0.11|

P, responders versus non-responders at 12 months.
inflammatory processes and subsequently persistent efforts to resolve inflammation made the serum HP levels increased at baseline and sustained up to 3 months in certain RA patients who have poor disease control by MTX.

Several limitations should be keep in mind when interpreting the current data. First, although we identified a potential association between HP serum levels and response of MTX treatment in RA patients, the precise nature of the mechanisms involved in this association remain unclear. It is possible that the efficacy of other DMARD therapies is also related to serum HP levels, although we have failed to confirm the association of HP serum levels with hydroxychloroquine therapy in a primary study. Second, the 12 weeks follow-up is a short time-frame to reveal a real association between HP and MTX response. Only 16 patients who had a significant change in HP levels at week 12 were followed up to 1 year. We found most of them (12/16) could maintain response at 1 year. However, a larger sample size and longer follow-up time is necessary to confirm the validity for prediction of MTX treatment response in RA. In addition to disease activity, other outcomes such as radiological progression of MTX therapy should be considered. Our data have indicated that the change in HP levels at 3 months could not predict the structural damage at 1 year. However, given the small sample size (n = 27) and the highly skewed SHS data in the current study, this preliminary conclusion needs confirmation.

In conclusion, we observed that serum levels of HP at baseline were related to response of 12 weeks MTX treatment in recent onset RA patients. Despite small patient numbers, the present data suggest for the first time that serum HP levels may potentially help predict response to MTX in RA. Further large, prospective and multicenter studies with longer follow-up times are needed to determine HP as a biomarker to predict MTX therapeutic response or as a target for treatment in RA.

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DISCLOSURES

None.

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