

Predicting the effectiveness of methotrexate therapy based on basal expression of the AMP-activated protein kinase gene in the blood of patients with rheumatoid arthritis

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Objective: to search for an association between basal expression of the AMP-activated protein kinase (adenosine monophosphate-activated protein kinase, AMPK) gene in the blood of patients with rheumatoid arthritis (RA) and disease activity, as well as joint destruction before and after 24 months of methotrexate (MT) therapy.

Patients and methods. The study included 40 patients with RA who met the 1987 ACR classification criteria, with a disease duration of not more than 2 years, with a mean age of 47.5 ± 15.5 years. All patients received 15 mg/week of MT. The number of swollen joints (SJC) out of 44, the number of tender joints (TJC) out of 53, the duration of morning stiffness (in minutes) and RA activity were assessed using the DAS28 index. The concentration of CRP and IgM rheumatoid factor (RF), antibodies to cyclic citrullinated peptide (ACCP) were assessed. To evaluate the radiological progression of RA, the Sharp method modified by van der Heijde was used. Gene expression in peripheral blood cells was determined by reverse transcriptase reaction and real-time polymerase chain reaction.

Results and discussion. During MT therapy, there was a decrease in disease activity by DAS28 index, CRP level, duration of morning stiffness, SJC and TJC. After 2 years, there were no statistically significant changes in the number of erosions, while the number of joints with narrowing of the joint space increased by the end of the study ($p=0.004$). Treatment with MT resulted in a decrease in AMPK expression. At the same time, patients who achieved remission had the highest level of AMPK before therapy, as did seronegative patients compared to seropositive ones. Based on the ROC analysis, the threshold value of AMPK gene expression was determined, which makes it possible to predict a high probability of a positive effect of MT therapy.

Conclusion. AMPK gene expression is associated with disease activity. Its high initial blood level in RA patients is associated with greater efficiency of MT and, possibly, plays a protective role. With an AMPK gene expression index greater than 3.83, the probability of a positive response to MT is high.

Key words: rheumatoid arthritis; gene expression; AMP-activated protein kinase gene; peripheral blood; inflammation; joint damage; methotrexate.

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Rheumatoid arthritis (RA) is the most common autoimmune rheumatic disease, which is based on a complex systemic multifactorial inflammatory process that leads to joint destruction [1]. Activation of T lymphocytes during the course of the disease causes the production of a wide range of pro-inflammatory cytokines, belonging mainly to the superfamilies of tumor necrosis factor (TNF) and interleukins (IL). Currently, drugs have been developed for the treatment of RA that specifically inhibit the production of various pro-inflammatory cytokines [2]. The effectiveness of therapy is assessed by the frequency of achieving low activity ($2.6 < \text{DAS28} < 3.2$) or remission ($\text{DAS28} < 2.6$) of the disease. The effect of the therapy is considered good when ΔDAS28 (initial DAS28 – DAS28 at the end of therapy) is > 1.2 [3]. However, in RA, the optimal strategy for predicting individual response to treatment has not been developed. A decrease in the activity of the disease is not accompanied by a complete recovery, but only leads to the stabilization of the inflammatory process. In this regard, drugs with different mechanisms of action can affect the course of RA in different ways, which is associated with the initial indicators of its clinical and laboratory activity and radiological changes [4].

Methotrexate (MT) is the main basic anti-inflammatory drug (DMARD) with which treatment begins in most cases of RA [5]. In addition, MT is often prescribed in combination with other DMARDs and genetically engineered biologics. At the same time, MT is not able to equally effectively control the disease in all patients [6], which leads to loss of time and progression of RA. Therefore, today, in order to save money and improve the treatment of patients, approaches are being developed that allow, before prescribing a specific antirheumatic drug, to identify patients in whom it is possible to achieve a significant reduction in the disease activity.

In the previous studies, we have shown that the initial expression of the extracellular matrix resorption (MMP9) and autophagy (ULK1) genes is associated with RA activity. High basal expression of the central regulatory genes mTOR, p21, TGF β 1 and Runx2, as well as genes responsible for inflammatory activity (TNF α), is a predictor of a good response to MT and may have a protective value, at least in patients who have not previously received DMARDs and systemic therapy with glucocorticoids (GC) [7, 8].

However, the most reliable way to identify patients responding to treatment is to analyze the metabolic potential of immune system cells, and primarily, changes in the energy metabolism in blood cells in RA [9]. Recent studies have shown that energy conversion in cells is controlled by AMP-activated protein kinase (adenosine monophosphate-activated protein kinase, AMPK), which regulates the levels of the main energy substrate adenosine triphosphate (ATP), as well as pro-inflammatory activity of immune cells [10]. In particular, in RA, AMPK activation in synoviocytes was associated with a decrease in the production of pro-inflammatory cytokines and metalloproteinases [11, 12], and the suppression of its activation was associated with an increase in inflammation in the synovial tissue [13]. Previously, in studies on animal models of RA, it was found that MT can contribute to the accumulation of AMP (adenosine monophosphate) in endothelial cells and, consequently, the activation of AMPK [14]. But it is still unknown how basal AMPK activity in cells can influence the outcome of RA therapy. In addition, it is unclear whether there is an association between AMPK gene expression and response to MT treatment.

The aim of the study was to search for an association between basal AMPK gene expression in the blood of RA patients and disease activity, as well as joint destruction before and after 24 months of MT therapy.

Patients and methods

The study included 40 patients with RA who met the classification criteria of the American College of Rheumatology (ACR) 1987 [15], with a disease duration of no more than 2 years. Among them, there were 5 men and 35 women, whose mean age was 47.5 ± 15.5 years, and who did not receive DMARDs and systemic GC therapy before the inclusion in the study. The patients were treated at V.A. Nasonova Research Institute of Rheumatology in 2007–2008 under the program "RADIKAL" (registration number of the clinical trial – 0120.0810610). The study protocol was approved by the local ethics committee, and all patients signed informed consent.

The exclusion criterion was the presence of contraindications for the use of DMARDs in effective therapeutic doses.

All patients were prescribed MT at a dose of 10 mg/week. After 2 weeks of treatment, the dose of the drug was increased to 15 mg/week, and patients continued to receive it for 2 years. In addition to MT, 11 (27.5%) of 40 patients used methylprednisolone 8 mg/day. Each patient was followed up by the same rheumatologist, visits were made every 6 months.

The control group consisted of 26 randomly selected blood donors without autoimmune diseases and aggravated heredity, comparable in sex and age with the patients of the main group.

Clinical, laboratory and instrumental methods of examination. The number of swollen joints (NSJ) out of 44, the number of painful joints (NPJ) out of 53, the duration of morning stiffness (minutes) and RA activity were assessed using the DAS28 index.

The concentration of CRP and IgM of rheumatoid factor (RF) in blood serum was determined by the immunonephelometric method on an automatic analyzer BN-100 (Dade Behring, Germany). The level of antibodies to cyclic citrullinated peptide (ACCP) was studied by enzyme immunoassay using a commercial kit from Axis-Shield Diagnostic Limited (Great Britain) according to the manufacturer's instructions.

All patients underwent x-rays of the hands and distal feet in direct projection. To assess the radiological progression of RA, the

Sharp method modified by van der Heijde [16] was used. At the same time, the number of erosions and narrowing of the joint spaces was counted in 16 joints and bones of each hand and in 6 joints of each foot. Indices of the number of erosions and narrowing of the joint space were recorded for each hand and each foot, calculating the average value of the estimates made by two researchers.

Molecular biological methods. Total RNA was isolated from whole blood using a commercial RIBO-sol-A kit (InterLabService, Moscow). For the reverse transcriptase reaction, the Reverta commercial kit (InterLabService, Moscow) was used. Real-time polymerase chain reaction was performed according to the previously described method [8] on an Applied Biosystems model 7300 instrument using gene expression kits (Applied Biosystems, USA): AMPK (Hs01562315_m1). β -actin served as endogenous control.

Clinical, immunological, and molecular biological parameters were recorded before and after 24 months of MT therapy.

Statistical analysis. Statistica software package (version 6.0 StatSoft) was used for statistical analysis. Quantitative features are presented as median and interquartile interval (Me [25th; 75th percentile]). Analyses were performed in duplicate. The results were statistically processed using the Mann–Whitney and Wilcoxon tests. The relationship of features was assessed using Spearman's rank correlation test (r). To assess the accuracy of the test, an analysis of ROC curves was carried out. Differences were considered statistically significant at $p < 0.05$.

Results

The average duration of RA was 7.8 ± 6.0 months. Fourteen (35.0%) out of 40 patients had a low level of ACCP, 25 patients (62.5%) were RF seropositive. Initially, the majority (62.5%) of patients had high disease activity ($\text{DAS28} > 5.1$). During the treatment, a significant decrease in RA activity, the duration of morning stiffness, NSJ and NPJ was noted ($p < 0.001$). After 24 months, 24 (60%) patients had moderate ($3.2 \leq \text{DAS28} \leq 5.1$), 4 (10%) patients had high ($\text{DAS28} > 5.1$) RA activity, and 12 (30%) patients had remission ($\text{DAS28} < 2.6$). At the beginning of the study, all patients had radiographic stage II of RA according to Steinbrocker. After 2 years, there were no statistically significant changes in the number of erosions, while the number of joints with joint space narrowing increased ($p = 0.004$). The dynamics of clinical, laboratory and radiological parameters is presented in Table 1.

Table 1. Dynamics of clinical, laboratory and radiological parameters in patients with early RA, Me [25th; 75th percentile]

	Before treatment (n=40)	After 2 years (n=40)	p
RF, ME/L	52 [9.5; 80]	25.1 [9.5; 58.9]	0.150
ACCP, U/L	48.8 [0.4; 100]	48.8 [0.4; 100]	0.996
CRP, mg/mL	14.7 [5.45; 24.7]	5.14 [2.49; 11.3]	0.002
DAS28	5.56 [5.04; 5.96]	3.29 [2.47; 3.82]	<0.001
Δ DAS28	–	2.45 [0.82; 3.32]	–
Stiffness, min	90 [30; 180]	15 [0; 30]	<0.001
NSJ	8 [5; 11]	1 [0; 3]	<0.001
NPJ	8 [6; 12]	2 [0; 6]	<0.001
Erosion number	0 [0; 1]	0 [0; 4.5]	0.07
Joint space narrowing	12 [7; 21]	18 [14; 28]	0.004

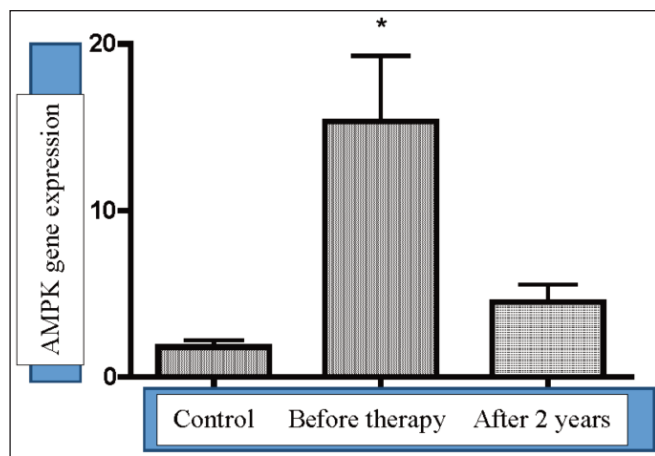


Fig. 1. Relative expression of the AMPK gene in the blood of RA patients before and after MT therapy ($n=40$) compared with healthy individuals (control, taken as 1). Here and in Fig. 2, 3: * - statistically significant differences compared to control; relative expression of the AMPK gene is expressed in relative units relative to the control

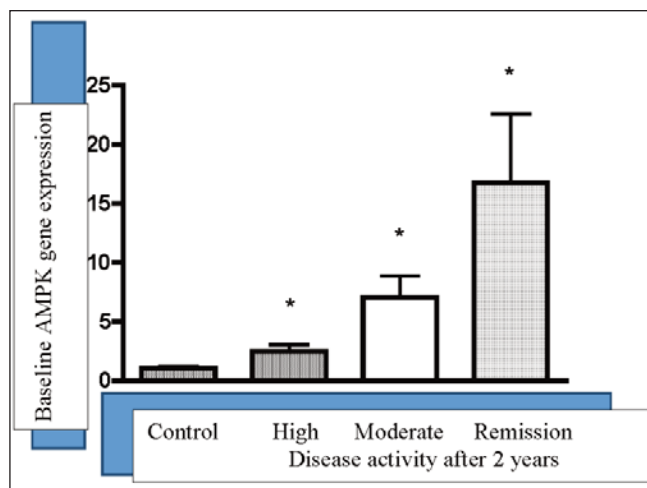


Fig. 2. Basal relative expression of the AMPK gene in healthy individuals (control) and in RA patients with high, moderate disease activity and remission, depending on the results of MT therapy

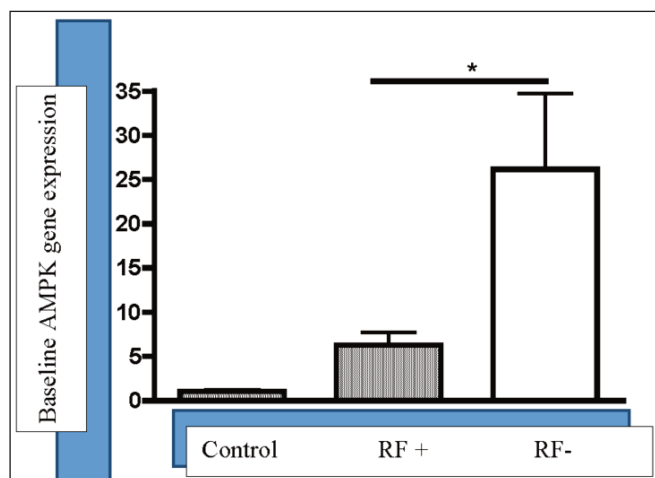


Fig. 3. Basal relative expression of the AMPK gene in seropositive ($n=25$) and seronegative ($n=15$) patients with RA

Evaluation of gene expression in the blood of RA patients before and after MT treatment. At the beginning of the study, RA patients had significantly increased expression of the AMPK gene compared to controls (Fig. 1).

Treatment with MT led to a significant decrease in AMPK gene expression, while it remained slightly higher than in healthy individuals. Evaluation depending on the results of MT therapy showed that individuals who achieved remission had a significantly higher initial level of the AMPK gene compared to the controls (Fig. 2) and patients with moderate RA activity, but the differences with the latter were not statistically significant.

Analysis of AMPK gene expression showed that in seronegative patients ($n=15$) it was significantly higher than in seropositive patients ($n=25$; Fig. 3).

Correlation analysis revealed a positive relationship between basal AMPK gene expression and Δ DAS28 and a negative relationship with RF and the duration of morning stiffness assessed after 2 years. On the contrary, AMPK gene expression at the end of the study positively correlated with the level of ACCP, the duration of morning stiffness, NPJ and NSJ, and DAS28, also assessed after 2 years (Table 2).

Joint space narrowing	–	–0.418 ($p=0.04$)
RF	–0.392 ($p=0.02$)	–
ACCP	–	0.459 ($p=0.024$)
DAS28	–	0.464 ($p=0.02$)
Stiffness, min	–0.399 ($p=0.02$)	0.486 ($p=0.016$)
NSJ	–	0.686 ($p=0.0001$)
NPJ	–	0.608 ($p=0.002$)

To assess the prognostic value of AMPK gene expression, an analysis of ROC curves was performed (Fig. 4), which confirmed a statistically significant relationship ($p=0.04$) between the initial level of the AMPK gene and the value of Δ DAS28: area under the curve (Area Under Curve, AUC) – 0.720, 95% confidence interval – 0.526–0.915.

To determine the sensitivity of RA patients to MT therapy, a binary linear classifier was used. The input data of this classifier were the AMPK gene expression before therapy, and the result of the classification was the assignment of the sample to one of two classes: the presence of sensitivity to therapy (Δ DAS28 > 1.2) or its absence (Δ DAS28 < 1.2). Next, the classification reliability parameters (sensitivity, specificity) were evaluated. The best prognostic accuracy in determining the response of a patient with RA to MT therapy was achieved at an AMPK gene expression level of 3.83 and maximum sensitivity (0.742) and specificity (0.778). Therefore, it is these indicators that make it possible to predict a high probability of a positive effect of MT treatment (Δ DAS28 > 1.2), while at the level of AMPK gene expression < 3.83 , the probability of a positive response is low.

Discussion

AMPK is the main regulator of metabolism, since its activation causes the phosphorylation of numerous enzymes, a decrease in the activity of anabolic pathways that consume ATP: the biosynthesis of fatty acids (FA), triglycerides, phospholipids and proteins, as well as the activation of catabolic pathways that generate ATP: glucose up-

ORIGINAL INVESTIGATIONS

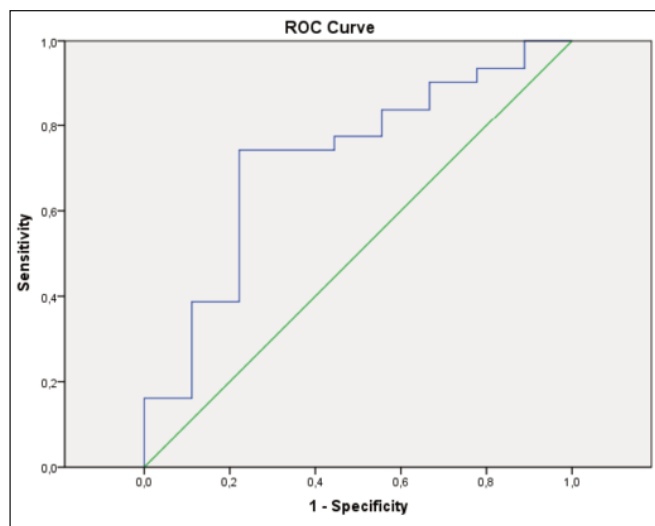


Fig. 4. Analysis of ROC-curves of the relative expression of the AMPK gene to predict the response of a patient with RA to MT therapy. AUC = 0.720; the best parameters of sensitivity – 74.2%, specificity – 77.8%

take and oxidation FA [17, 18]. In the long term, AMPK enhances its action by phosphorylation of transcription factors and co-activators that regulate gene expression, for example by decreasing the production of lipogenic enzymes and increasing the production of enzymes of mitochondrial oxidative metabolism and mitochondrial biogenesis. The anti-anabolic effect of AMPK is especially important in inhibiting the expression of mTOR, which suppresses the translation of proteins necessary for switching from oxidative phosphorylation to aerobic glycolysis, which is observed in rapidly growing cells. On the contrary, the loss of the AMPK gene promotes the development of aerobic glycolysis and cell proliferation [19]. In this regard, it is important to note that, according to the present study, high basal AMPK expression turned out to be a marker for predicting a positive response to MT treatment in RA patients who had not previously received DMARDs; this was also confirmed by the analysis of the ROC curve profile. The prognostic value of AMPK expression indicates the important role of energy metabolism in the suppression of RA.

In our study, the protective role of high AMPK expression is indicated by the negative relationship between its basal concentration and RF level and the duration of morning stiffness at the end of the observation since these indicators are markers of inflammation [4]. A positive relationship between AMPK expression after MT and DAS28 therapy, NSJ, NPJ, duration of morning stiffness may indicate the need to activate this gene to relieve inflammation and pain, since AMPK production decreases with low inflammation activity and pain severity. Our data are consistent with the results of other studies, which also showed that AMPK has an anti-inflammatory effect due to its effect on cellular metabolism [20]. Thus, in in vitro studies, the suppression of AMPK production in macrophages was accompanied by an increase in the level of pro-inflammatory cytokines in response to the action of fatty acids and lipopolysaccharides [21, 22]. In addition, a significant excess of AMPK gene expression in seronegative patients in our study confirms previously obtained data on a more severe course of the disease in seropositive RA patients [23, 24].

At the same time, unlike other drugs, including metformin, hormones (leptin, adiponectin), and nutraceuticals that activate AMPK [18, 19], MT reduced AMPK expression, according to the present study. When AMPK expression approaches the norm, it may indicate the restoration of the energy balance of cells during MT therapy.

Conclusion

Thus, AMPK gene expression is associated with RA activity. The high initial production of this gene in the blood of patients with this disease is associated with greater efficacy of MT and, possibly, plays a protective role. Therefore, based on the analysis of AMPK gene expression in RA patients who have not previously received DMARDs, it is possible to predict the response to MT therapy with a high probability.

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Conflict of Interest Statement

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