Expression of folylpolyglutamyl synthetase predicts poor response to methotrexate therapy in patients with rheumatoid arthritis

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Abstract

Objective

The enzyme folylpolyglutamyl synthetase (FPGS) is involved in the resistance to methotrexate in tumor cell lines. The aim of the present study was to determine the impact of FPGS mRNA expression on resistance to methotrexate therapy in patients with rheumatoid arthritis (RA).

Methods

We determined the expression of FPGS mRNA using the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in 141 patients with RA. All patients received methotrexate therapy. The primary outcome measures were disease activity as determined by a disease activity score (DAS) and response to therapy.

Results

Seventy-eight of 141 patients (55%) showed expression of FPGS mRNA. FPGS mRNA expression was not associated with age, sex, disease duration, white blood cell count, erythrocyte sedimentation rate, C-reactive protein (CRP), number of swollen joints, number of painful joints, and combined therapy with other disease-modifying antirheumatic drugs (DMARDs) or additional corticosteroids. The response rate to methotrexate therapy was 44% for the total study population. Patients without FPGS mRNA expression showed a significantly higher response rate than patients with FPGS mRNA expression (57% versus 33%; p = 0.005). Multivariate logistic regression analysis revealed that female sex (p = 0.009) and FPGS mRNA expression (p = 0.004) were independent predictive factors for failure to achieve a response to methotrexate therapy.

Conclusion

FPGS mRNA expression is an independent predictive factor associated with poor response to methotrexate therapy in RA patients.

Key words

Drug resistance, clinical outcome, polyglutamylation, PCR.

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Introduction

The antimetabolite methotrexate constitutes an important disease modifying drug (DMARD) in rheumatoid arthritis (RA) and has become the first therapeutic choice (1). Methotrexate finds its way into the cell via a carrier or a receptor-mediated pathway. Intracellularly up to five glutamate residues are added by the enzyme folylpolyglutamyl synthetase (FPGS). Because of their high charge, which prevents diffusion through the cellular membrane, methotrexate polyglutamates are retained longer intracellularly than the unconjugated compound. Both the amount and the chain length of polyglutamates are known to be important factors for the cytotoxicity of high-dose, short-term methotrexate exposure. Although there are many hypotheses with regard to the possible mode of action of low-dose methotrexate in RA, the exact mechanisms of action are still largely unknown. It has been reported that methotrexate exerts mainly antiinflammatory and antiproliferative effects on cultured differentiating myeloid monocytic cells (2, 3).

In comparison to other DMARDs, methotrexate therapy is known to be of longer duration; however, its use is limited by toxicity and inefficacy (4). The development of drug resistance is a limiting factor for methotrexate efficacy in patients with malignant disorders (5). Various mechanisms involved in the development of resistance to methotrexate have been studied in cancer cell lines (5). They include decreased influx of methotrexate via the reduced folate carrier (6), increased activity and expression of dihydrofolate reductase (DHFR) (7), decreased affinity of methotrexate to mutated DHFR (8), and the increased efflux of methotrexate by transport proteins such as the multidrug resistance protein family (MRP1-MRP3) (9). Another important factor for methotrexate resistance in cancer cell lines and malignant diseases is altered polyglutamylation due to alterations in the expression or activity of FPGS, an enzyme that plays a crucial role in cellular folate metabolism and in the pharmacology of antifolates, including methotrexate (10). FPGS is expressed in a variety of normal tissues including heart, skeletal muscle, liver, small intestine, lung and also in mononuclear cells of the peripheral blood (12). Altered polyglutamylation is an important mechanism of methotrexate resistance in both cancer cell lines and tumor samples and may also be responsible for resistance to the drug in patients with RA. To further pursue this possibility, we investigated a possible relationship of FPGS-conjugation, one of the most important factors of resistance, and methotrexate inefficacy in RA patients.

Patients and methods

Patients

One hundred and forty-one patients with RA according to the American College of Rheumatology classification criteria (11) and on treatment with methotrexate were enrolled in a study to assess the impact of FPGS mRNA expression on resistance to methotrexate therapy. Patients were treated at the Second Department of Medicine, Lainz Hospital, Vienna. All patients gave their written informed consent, and the local Ethics Committee approved the study protocol.

Methotrexate was administered orally once a week at a mean dosage of 12 mg/week (5–20 mg) without folate supplementation. The methotrexate dosage was adjusted based on the occurrence of adverse effects and the patient's disease activity. The patients' characteristics are summarized in Tables I and II. Clinical assessment included the white blood cell count, erythrocyte sedimentation rate, C-reactive protein (CRP), the number of swollen joints, and the number of tender joints.

Disease activity was defined using the disease activity score (DAS28) (13,14) and then categorized into inactive (DAS28 < 3.2), moderately active (3.2 ≤ DAS28 ≤ 5.1), and highly active disease (DAS28 > 5.1). Treatment efficacy was determined using response criteria based on the DAS28 (14). Disease activity was calculated at baseline and at the time of sampling to determine the response. A good response was defined as a decrease in DAS > 1.2 and a

Table I. Baseline characteristics of 141 patients and FPGS mRNA expression.

	No FPGS mRNA expression (n = 63)	FPGS mRNA expression (n = 78)	p-value
Age (years)	60 (30-98)	59 (29-83)	0.5†
Patients ≥ 60 years	31 (49%)	36 (46%)	0.7‡
Female sex	48 (76%)	62 (80%)	0.6‡
Disease duration (months)*	19 (1-121)	25 (0-121)	0.9†
White blood cell count*	8600 (5080-15700)	7550 (4900-20100)	0.3†
Erythrocyte sedimentation rate (mm/h)*	36 (8-110)	43 (4-100)	0.2†
C-reactive protein (mg/l)*	18 (3-109)	25 (2-166)	0.03†
Number of swollen joints*	8 (0-28)	6 (0-23)	0.6†
Number of painful joints*	8 (0-28)	6 (0-26)	0.2†
Disease activity score*	5.1 (3.3-7.6)	5.0 (3.2-8)	0.4†
Disease activity			
Moderate	32 (51%)	43 (55%)	0.6‡
High	31 (49%)	35 (45%)	

* Median (range); † Mann-Whitney U test; ‡ Chi-square test.

Table II. Characteristics of 141 patients at the time of sampling and FPGS mRNA expression.

	No FPGS mRNA expression (n = 63)	FPGS mRNA expression (n = 78)	p-value
White blood cell count*	7900 (2650-15700)	7080 (2980-15400)	0.2†
Erythrocyte sedimentation rate (mm/h)*	22 (4-80)	24 (4-92)	0.1†
C-reactive protein (mg/l)*	8 (3-77)	10 (3-139)	0.4†
Number of swollen joints*	1 (0-22)	2 (0-19)	0.5†
Number of painful joints*	1 (0-25)	2 (0-28)	1.0†
Disease activity score*	3.4 (1.2-6.8)	3.7 (1.2-6.1)	0.3†
Other DMARDs¶			
Chloroquine	8 (13%)	11 (14%)	1.0‡
Sulphasalazine	1 (2%)	2 (3%)	
Cyclosporin A	1 (2%)	1 (1%)	
Corticosteroids	41 (65%)	53 (68%)	0.7‡
Disease activity			
Inactive	26 (41%)	27 (35%)	0.7‡
Moderate	29 (46%)	38 (49%)	
High	8 (13%)	13 (16%)	
Response			
No	27 (43%)	52 (67%)	0.005‡
Yes	36 (57%)	26 (33%)	

* Median (range); † Mann-Whitney U test; ‡ Chi-square test; ¶ disease-modifying antirheumatic drugs.

decrease in the disease activity category, a moderate response as a decrease in DAS of 0.6 – 1.2 and a decrease in the disease activity category, and no response as a decrease in DAS < 0.6 or no change in disease activity (14). For further analyses, patients were divided into responders (patients with good response) and non-responders (patients with no or only a moderate response).

RT-PCR analysis

Mononuclear cells of the patients were isolated by density gradient centrifugation (Ficoll-Paque Plus, Amersham Pharmacia Biotech, Uppsala, Sweden) within 24 hours after sampling and stored at -20°C until use. Total RNA was isolated using an RNA isolation kit (RNEasy, Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

cDNA synthesis with 1 µg total RNA as template was performed using Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD, USA) following the manufacturer's recommendations.

The cDNA reaction mixture (2 µl) was used for amplification of specific DNA sequences in the presence of 1x *Taq* DNA polymerase buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate together with 1 mM of each primer, and 2.5 units *Taq* DNA polymerase (Promega, Madison, WI, USA) in a total volume of 50 µl. Thirty five cycles at 96°C for one minute, 65°C for one minute, 72°C for one minute, and quick chilling to 4°C in a 9600 thermal cycler (Perkin Elmer, Emeryville, CA, USA) were carried out. The last cycle included an additional elongation step at 72°C for 7 minutes. The following primers were used to amplify the FPGS gene (15): Forward primer FPGS-241 (5'-AGCCCGGACCTCT GGAGTG-3', bases 1321-1339), reverse primer FPGS-242 (5'-CTGCCAGTG ACTAGCACATGG-3', bases 1563-1583). The primers yielded a product of 263 bp. The human HL60 promyelocytic leukemia cell line was used as positive control for FPGS expression (16). RT-PCR with primers for the β_2-microglobulin gene was performed as control for the integrity of the cDNA. Negative controls containing aliquots of cDNA reaction mixtures prepared without the addition of RNA were included in each experiment.

RT-PCR products were separated by electrophoresis on 2% agarose gels containing 0.5 mg/l ethidium bromide for DNA visualization. Gel photography and storage of the photographs were performed using E.A.S.Y Win32 software (Herolab, Wiesloch, Germany).

Statistical analysis

Associations of FPGS mRNA expression with clinical and laboratory parameters including response to methotrexate therapy were assessed by chi-square tests. For comparison of continuous variables between groups, Mann-Whitney U tests were performed. To describe the unadjusted effects of covariates on response rates, univariate logistic regression analysis was performed. Multiple logistic regression models were used to assess the independent effect of FPGS mRNA expression on response rates. All p-values are the results of two-sided tests. All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA).

Results

Expression of FPGS

FPGS mRNA expression was assessed at the time of sampling using RT-PCR in 141 patients with rheumatoid arthritis receiving methotrexate therapy. Expression of FPGS mRNA was observed in 78 patients (55%). To examine any possible changes in FPGS mRNA expression during methotrexate therapy, we analysed consecutive samples of various patients (both responders and non-responders) at different times (n = 16, time between samples 105 – 732 days). No significant differences in FPGS mRNA expression were found (data not shown). In addition, FPGS mRNA expression was also observed in control samples of healthy individuals (data not shown.)

FPGS and clinical parameters

The major clinical and laboratory parameters at baseline and at the time of sampling are summarized in Tables I and II. The baseline level of CRP was higher in patients with FPGS mRNA expression than in those without (median 25 mg/l versus 18 mg/l; p = 0.03). Patients with or without FPGS mRNA expression did not show differences in age, sex, disease duration, white blood cell count, erythrocyte sedimentation rate, swollen joint count, tender joint count or overall disease activity. Combination therapy with other DMARDs and/or additional corticosteroids was also equally distributed between patients with or without FPGS mRNA expression (Tables I and II).

We observed a relationship between clinical or laboratory parameters and disease activity at the time of sampling. Patients with active disease were more often female (88% versus 62%; p < 0.001), and showed elevated CRP levels (median 13 mg/l versus 5 mg/l; p < 0.001) and a higher white blood cell count (median 7955 versus 6700; p = 0.004) at the time of sampling compared to patients with lower disease activity. Treatment with additional coticosteroids (p = 0.002) and combination therapy with other DMARDs (p= 0.001) were also more frequent in the active disease group (data not shown).

FPGS and response

All 141 patients were treated with methotrexate. Response was defined using a disease activity score as described above. The response rate of the total study population was 44%. Patients without FPGS mRNA expression showed a significantly higher response rate than patients with FPGS mRNA expression (57% versus 33%; p = 0.005) (Table II, Fig. 1). Patients with response to methotrexate therapy had lower CRP levels at the time of sampling than non-responders (p < 0.001, data not shown). No other clinical or laboratory parameter was associated with the response to methotrexate therapy.

We also performed logistic regression analyses including age, sex, CRP, additional therapy with corticosteroids, therapy with other DMARDs, and FPGS mRNA expression. In the univariate analysis, the odds ratios for failure to achieve a response were 1.3 for age ≥ 60 years (p = 0.4), 3.5 for female sex (p = 0.03), 1.0 for CRP > 20 mg/l (p = 0.9), 2.0 for additional therapy with corticosteroids (p = 0.06), 2.2 for therapy with additional DMARDs (p = 0.1) and 2.7 for FPGS mRNA expression (p = 0.005) (Table III). In the multivariate analysis, the odds ratios were 1.6 for age ≥ 60 years (p = 0.2), 3.3 for female sex (p = 0.009), 1.4 for CRP > 20 mg/l (p = 0.4), 1.9 for additional therapy with corticosteroids (p = 0.1), 1.7 for therapy with additional DMARDs (p = 0.3) and 3.0 for FPGS mRNA expression (p = 0.004) (Table III). Thus, FPGS mRNA expression had an independent effect on response to methotrexate therapy.

Discussion

In our study, we demonstrated that expression of FPGS mRNA in mononuclear cells predicts a poor response to methotrexate therapy in RA patients. This effect was independent of other factors, such as age and additional therapy with corticosteroids or other DMARDs. Female sex was also of independent predictive value for a poor response to methotrexate therapy.

Sex has been proven to be a prognostic and predictive factor in a large clinical trial (17). Interestingly, it has been reported recently that the percentage of apoptotic synovial macrophages increased under methotrexate and testosterone compared to methotrexate or

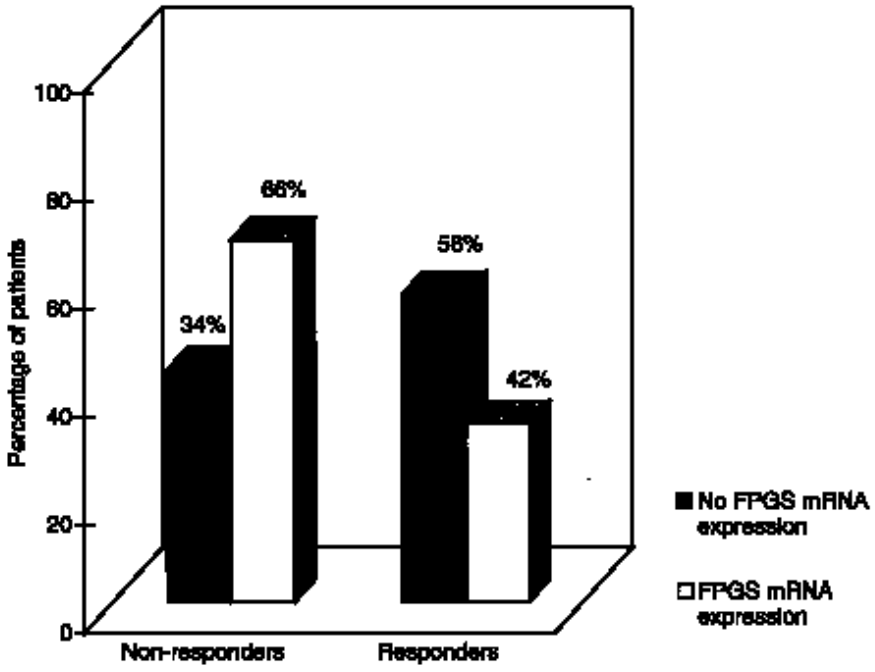

Fig. 1. FPGS mRNA expression and response to methotrexate therapy. FPGS mRNA expression and response to methotrexate therapy were assessed as described in Patients and Methods. Expression of FPGS mRNA was more frequently observed in non-responders than in responders (p = 0.005).

Table III. Logistic regression analysis for failure to achieve a response to methotrexate therapy.

	Univariate			Multivariate		
	Odds ratio	95% CI *	p-value	Odds ratio	95% CI	p-value
Age ≥ 60 yr	1.3	0.5-2.6	0.4	1.6	0.7-3.3	0.2
Female sex	3.5	1.5-8.3	0.03	3.3	1.4-8.2	0.009
CRP > 20 mg/l †	1.0	0.5-2.0	0.9	1.4	0.7-3.0	0.4
Corticosteroids	2.0	1.0-4.0	0.06	1.9	0.9-4.0	0.1
DMARDs ‡	2.2	0.8-5.6	0.1	1.7	0.6-4.8	0.3
FPGS mRNA expression ¶	2.7	1.3-5.3	0.005	3.0	1.4-6.2	0.004

* CI: confidence interval; † CRP: C-reactive protein; ‡ DMARDs: disease-modifying antirheumatic drugs; ¶ FPGS: folylpolyglutamyl synthetase.

testosterone alone, therefore suggesting a beneficial effect of male hormones on the response to methotrexate therapy (3). Additional corticosteroids or DMARDs were preferentially given to patients who had failed to respond or had shown an incomplete response to methotrexate. However, the response rate of these patients was similar to patients who were treated with methotrexate monotherapy.

FPGS is responsible for the polyglutamylation of antifolates. These polyglutamates are retained longer in cells and therefore contribute to the cytotoxicity of methotrexate in cancer therapy to a high extent. The exact mechanism of methotrexate action in RA still needs to be elucidated. It has been postulated that inhibition of 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, an enzyme of purine-biosynthesis, occurs in addition to the inhibition of DHFR. This leads to a decreased purine biosynthesis and to an increased release of adenosine inhibiting the migration of neutrophil granulocytes (2,18,19). The synthesis of polyamines seems to be involved in an alternative mechanism. Polyamines are important for the immune response and their synthesis depends greatly upon their precursors such as methionine. The intracellular methionine pool is filled via homocysteine. This constitutes a folate-dependent process and is consequently inhibited by methotrexate. Furthermore, it has been postulated that methotrexate may interfere with the activity of proinflammatory cytokines in the synovial compartment and consequently may reduce disease progression (20, 21). Methotrexate is also able to exert indirect inhibitory effects on the synthesis of the inflammatory enzyme cyclooxygenase-2, the chemotaxis of neutrophils, and on the production of synovial matrix metalloproteinase via the modulation of cytokines and stimulates the production of metalloproteinase inhibitors (22).

Most studies regarding FPGS-mediated resistance to methotrexate have dealt with either tumor cell lines or clinical samples from malignant diseases. Decreased polyglutamylation via decreased expression or activity of FPGS was identified as a mechanism of resistance to high-dose, short-term methotrexate exposure in human leukemia (23-27) and squamous cell carcinoma cell lines (28). Resistance to methotrexate due to altered polyglutamylation has also been reported in leukemia. In methotrexate sensitive B-lineage acute lymphoblastic leukemia, FPGS mRNA expression and activity were higher than in methotrexate resistant T-lineage acute lymphoblastic leukemia (29, 30).

Our finding raises the possibility that the role of FPGS expression in the resistance to methotrexate in RA may be different to that in malignancies. Methotrexate polyglutamylation may not play the pivotal role in its efficacy in RA as compared to malignant diseases. Angelis-Stoforidis *et al.* reported that intracellular levels of MTX polyglutamates in mononuclear cells of patients with RA were not associated with response, whereas higher levels in erythrocytes and polymorphs correlated with better clinical efficacy in patients (31). Interestingly, methotrexate resistant human T-lymphoblastic leukemia CCRF-CEM cells with decreased polyglutamylation exposed to methotrexate for 120 hours lost their resistance and became as sensitive as the parental sensitive cells (24). However, altered polyglutamylation due to mutations or changes in protein synthesis may still be an important mechanism of MTX resistance in patients with RA showing FPGS mRNA expression, as already observed in both cancer cell lines and tumor samples.

Development of resistance may be associated with changes in the maturation and proliferative capacities of the lymphocytes, which are the main mediators of the immune response. Barredo *et al.* found (32) that FPGS levels in rats were high during embryogenesis and dropped when maturity was reached. Similar results were seen during the differentiation of human HL-60 tumor cells. FPGS activity was very low in mature human hematopoietic cells, in contrast to the high activity of FPGS in lymphoblasts of patients with ALL (12).

It has been reported that immature differentiating monocyte function is the main target of methotrexate in RA (3). The antiproliferative activity of methotrexate may be mediated by cell apoptosis and its antiinflammatory effect may be due to cytokine inhibitor release, as demonstrated on cultured differentiating myeloid monocytic cells. Methotrexate treatment did not influence the growth or apoptosis of differentiated mature synovial macrophages (3), but might inhibit the recruitment of immature and inflammatory monocytes from bone marrow into the sites of inflammation. Therefore, the number of immature differentiating monocytes may be lower in patients with a good response to methotrexate therapy. In this context the rapid decline of FPGS activity and gene expression during maturation in leukemic HL60 cells has to be mentioned (16).

In conclusion, FPGS mRNA expression in mononuclear cells may be a marker for resistance to methotrexate therapy in patients with RA and may be a target for future treatment strategies to improve the clinical outcome in RA.

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