Overexpression of P-glycoprotein on fibroblast-like synoviocytes in refractory rheumatoid arthritis patients: a potential mechanism for multidrug resistance in rheumatoid arthritis treatment

Y.M. Liu[1], J.W. Chen[1], L.X. Chen[2], X. Xie[1] and N. Mao[1]

[1]Department of Rheumatology Immunology,
The Second Xiangya Hospital of Central South University, Changsha,
Hunan Province, China
[2]Department of Rheumatology Immunology, The First Hospital of Changsha,
Changsha, Hunan Province, China

Corresponding author: J.W. Chen
E-mail: jinwei7310@163.com

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ABSTRACT. This study aims to investigate the role of P-glycoprotein (P-gp) expression level in drug resistance to disease-modifying anti-rheumatic drugs in refractory rheumatoid arthritis (RRA). We evaluated and compared the expression levels of P-gp in fibroblast-like synoviocyte (FLS) cells in patients with rheumatoid arthritis (RA) and osteoarthritis (OA), and investigated the potential mechanism of P-gp-induced multidrug resistance in RRA. Ten patients were enrolled and divided into two groups: six in the RA group and four in the OA group. The expression level of P-gp in FLS cells was detected by western blotting following cell culture. A linear correlation algorithm was used to assess the association between the level of P-gp and disease activity

(using DAS28 scoring), as well as the duration of methotrexate (MTX) treatment in the RRA patients. The level of P-gp in the RRA patients was markedly higher than that in the OA patients ($P < 0.05$, $t = -4.179$). There was a positive linear correlation between the P-gp level in FLS cells and the duration of MTX treatment in the RRA group ($\Gamma = 0.733$, $P < 0.05$), whereas there was no significant correlation between the P-gp level and DAS28 scoring ($\Gamma = 0.206$, $P > 0.05$). P-gp might be upregulated during the progression of RRA, which possibly correlates with the development of resistance to MTX.

Key words: Multidrug resistance; P-glycoprotein; Refractory rheumatoid arthritis; Fibroblast-like synoviocytes

INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease that is characterized by chronic progressive polyarthritis, and it mainly affects facet joints. The most important pathologic feature of RA is inflammation within the synovial tissues, followed by excessive proliferation of synovial cells. Progression of RA results in severe disability and pain (Harris, 1990). It has been reported that the disability rate reaches 60% in 5-10 years in the natural course of RA progression (Allaire et al., 2008). Disease-modifying anti-rheumatic drugs (DMARDs) are widely used for preventing and controlling abnormal inflammation, erosive synovitis, and joint destruction. Despite a better prognosis of RA using DMARDs, the symptoms fail to improve in certain RA patients after treatment for 6 months with two or more types of DMARD. It has been noted that the incidence of severe bone destruction increases along with the ongoing medical treatment in these patients. Clinically, these patients are typically diagnosed with refractory rheumatoid arthritis (RRA). Apart from the pathogenesis of RA, drug resistance to DMARDs may also contribute to the development of RRA, but the mechanism remains elusive.

The overexpression of P-glycoprotein (P-gp) is one of the many mechanisms proposed for multidrug resistance (MDR). P-gp, a 170-kDa product of the *ABCB1* gene, has emerged as the one of the major molecules associated with MDR and poor prognosis during chemotherapy for various malignancies (Beck et al., 1996; Linn et al., 1996; Advani et al., 1999; List et al., 2002). P-gp is a member of ATP-binding cassette transporter superfamily and functions as an energy-dependent transmembrane efflux pump. It is expressed in normal cells and tissues, especially at the blood-brain barrier, and in the digestive tract, liver, renal proximal convoluted tubules, adrenal glands, peripheral lymphocytes, hematopoietic stem cells of $CD34^+$, etc. (Fojo et al., 1987; Cordon-Cardo et al., 1989; Cordon-Cardo et al., 1990). As an ATP-dependent efflux pump, P-gp is responsible for actively pumping hydrophobic substances out of cells. Thus, P-gp plays a significant role in physiological detoxification, given its function of eliminating excess toxic materials in cells, including drugs and their metabolites (Gerlach et al., 1986; Gottesman and Pastan, 1993). Anti-tumor chemical drugs can be transferred out of tumor cells by P-gp, which affects the tumor's response to these drugs. Similarly, when used for treating RA, DMARDs such as methotrexate (MTX), and gold agents can also be pumped out of cells by P-gp, (Gottesman and Pastan, 1993). Hence, P-gp acts as a transporter of certain substances and drugs in cells.

Recently, the mechanism underlying MDR has been frequently studied in tumors but rarely in RA, with only a few studies focusing on MDR and RA. Morgan et al. (2003) reported that MDR occurred in approximately 5% (13 out of 256) of patients with RA. RA patients express higher levels of P-gp on peripheral blood lymphocytes compared with their counterparts without RA (Maillefert et al., 1996). Likewise, the synovium tissue expresses higher levels of P-gp mRNA in RA patients, especially in those treated with three types of DMARD (Jorgensen et al., 1995). Th1 cells also exhibit increased expression of P-gp and participate in drug resistance to sulfasalazine and bucillamine in RA patients, which suggests P-gp as an indicator of response to these two DMARDs (Marchetti et al., 2007). Consequently, we infer that P-gp is involved in drug resistance to DMARDs in RRA.

Synovium cells predominantly produce multiple cytokines, such as IL-1, IL-6, and TNF-α, thereby mediating cellular immune responses, and are involved in the progression of chronic inflammatory diseases. These cytokines have been proven to act as key effectors contributing to persistent inflammation in RA (Bartok and Firestein, 2010). Furthermore, the synovium is an emerging therapeutic target for treating RA clinically. Therefore, we hypothesize that the elevated expression of P-pg in fibroblast-like synoviocyte (FLS) cells in RRA patients may correlate with MDR, leading to RRA.

MATERIAL AND METHODS

Study population

Synovial tissue samples (RA and OA) were collected from ten patients during total knee replacements at the Department of Osteology of the Second Xiang Ya Hospital of Central South University. RA synovial tissues were obtained from six RA patients who developed high resistance to DMARDs and aggravated joint destruction. OA synovial tissues were obtained from four OA patients at the advanced stage of OA. All RA patients met the 1987 revised American College of Rheumatology (ACR) classification criteria for RA (Arnett et al., 1988). Demographic data including patients, gender, age, duration of disease, P-gp, DAS28 score, and duration of MTX are presented in Table 1.

Table 1. A list of RRA patients with three measured values: expression of P-gp in FLS, DAS28 score and length of MTX medication in RRA. Relative optical density of P-gp was obtained by the density value of P-gp and GADPH read by software, and DAS26 is a clinical scoring for evaluating RA disease status. Duration of MTX was the time period of MTX administration.

No.	Gender	Age	Duration of disease (year)	P-gp (relative optical density)	DAS28 score	Duration of MTX (months)
1	Male	61	8	0.376	5.4	36
2	Female	58	13	0.681	7.2	42
3	Female	72	12	0.342	5.8	35
4	Male	70	9	0.717	4.8	48
5	Female	56	7	0.430	5.2	32
6	Female	63	8	0.373	5.6	28

The DAS28 score was calculated by evaluating disease activity (http://www.das-score.nl/www.das-score.nl/).

To avoid the effect of steroids on cells, patients who had received or required prednisolone during the study were excluded. Cyclosporin A administration was also excluded because the drug is a P-glycoprotein inhibitor and may "protect" drug resistance (Maillefert et al., 2000). Informed consent was signed by all patients enrolled in this investigation. All study

procedures were in accordance with the ethics committee of The Second Xiang Ya Hospital of Central South University.

Materials

Synovial knee tissues were provided by the Department of Osteology of the Second Xiang Ya Hospital of Central South University. The main reagents for cell culture were Dulbecco's modified Eagle's medium (DMEM; 4.5 g/L), collagenase I and bovine serum albumin (2%) (Sigma-Aldrich, St. Louis., MO, USA), rat polyclonal P-gp antibody (Abcam Plc.), 2.5% trypsin, and Tris-buffered saline (TBS) adjusted to pH 7.4 at a concentration of 2% (Central Laboratory of the Second Xiang Ya Hospital, China).

Cell culture

Two-step *in situ* collagenase perfusion is widely used for digestion (Crofford et al., 1997), and was applied in our study to isolate synovial cells from synovial tissues to quantitatively measure the expression levels of P-gp in synovial cells. Human FLS cells were routinely grown in 75 cm^2 plastic flasks and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The treated cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. At approximately 80% cell confluence, the cells were trypsinized, harvested, and redistributed for subsequent cell culture. FLS cells were used between the third and fifth cell passage, and were assessed for $CD3^-CD14^-CD90^+$ by flow cytometry.

Western blotting

The FLS cells were lysed for subsequent western blotting. Cells at 80% cell confluence were lysed for 5 min on ice in lysis buffer. Total cell lysates were sonicated briefly and centrifuged for 10 min at 14,000 *g* at 4°C. An aliquot was removed to determine the concentration of proteins. Equal amounts of protein samples were separated by 8% and transferred to a polyvinylidene fluoride (PVDF) membrane (0.22 mM) using a transfer current of 250 mA at 4°C for 11 h. Membranes were blocked for 1 h at room temperature with 10% non-fat dry milk in TBST solution (TBST is a mixture of Tris-buffered saline and Tween 20). The membranes were incubated with rat polyclonal P-gp antibodies (1:300) in TBST overnight at 4°C. After washing the membranes three times with TBST (10 min each time), they were incubated with rat anti-rabbit antibodies (1:5000) in TBST for 2 h at room temperature, and washed again with TBST. A commercial kit was used to detect target proteins by chemiluminescence. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control. The developed films were scanned and the optical density (OD) values were quantified using the Quantity One software of a GS-800 calibrated densitometer. The relative OD density was calculated by dividing the value for P-gp by that for GAPDH based on the software reading.

Statistics

The SPSS software version 17 was used for statistical calculations. Data are reported as means ± standard deviation. The means of OD were calculated to compare the mean data

between the RRA and OA groups. An independent sample *t*-test assuming unequal variances was performed to compare the mean values between two groups. Linear correlation analysis was used to investigate the correlation between MTX and DAS28 score in the RRA group. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of expression of P-gp in FLS between RRA and OA patients

The expression levels of P-gp in FLS from six RRA patients and four OA patients were quantitatively analyzed. The average OD values in the RRA and OA groups were 0.486 ± 0.167 and 0.193 ± 0.031, respectively. An independent sample *t*-test was used to compare the mean OD values between the two groups owing to the data assuming unequal variances ($F = 13.41$, $P < 0.01$). As shown in Figure 1, the expression level of P-gp in the RRA group was significantly higher than in the OA group ($P < 0.05$, $t = -4.179$).

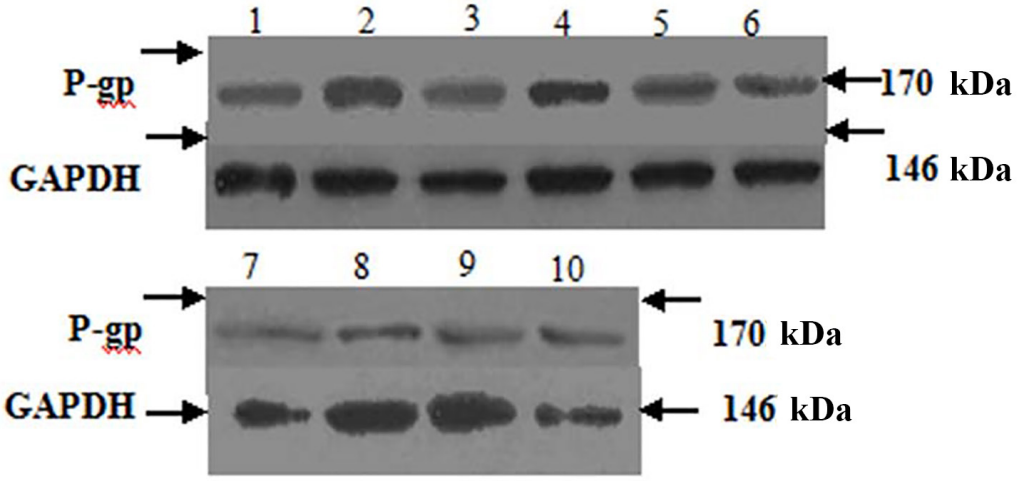

Figure 1. Western blot of fibroblast-like synoviocytes (FLS) cells isolated from refractory rheumatoid arthritis (RRA) patients (*lanes 1-6*) and osteoarthritis (OA) patients (*lanes 7-9*). FLS cells were collected from synovial tissues and cultured *in vitro*, followed by cell harvesting. The expression levels of P-glycoprotein (P-gp) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) of the cell lysates were assessed by western blotting.

Correlation between P-gp level and duration of MTX therapy

The relationships between disease activity, duration of MTX treatment, and expression of P-gp in the FLS cells from the RRA patients were assessed reciprocally via linear correlation analysis. DAS28 scoring was performed to evaluate disease activity in the RRA patients. As shown in Tables 1 and 2, positive correlation was observed between P-gp levels and duration of MTX treatment in the FLS cells from RRA patients ($\Gamma = 0.733$, $P < 0.05$), whereas no significant correlation existed between the P-gp level in the FLS cells and the DAS28 score ($\Gamma = 0.206$, $P > 0.05$), suggesting an association between upregulated expression of P-gp in FLS cells and longer duration of medication.

Table 2. Correlation of expression of P-gp in RRA group about disease activity and medication period of MTX.

Variables	Γ	P
DAS28 score and expression of P-gp	$\Gamma_1 = 0.206$	$P_1 = 0.695$
Time of MTX treatment and expression of P-gp	$\Gamma_2 = 0.733$	$P_2 = 0.039$

DISCUSSION

RA is a chronic autoimmune joint disease that is characterized by chronic inflammation and pannus formation inside the joint synovium. The complex pathogenesis of RA involves a variety of immune cells, inflammatory cytokines, and some related proteases. An intricate interaction network triggers synovial proliferation and chronic inflammation. FLS cells in the synovial intimal lining also play a key role in producing cytokines to maintain inflammation and proteases for cartilage destruction (Brentano et al., 2005; Sawai et al., 2005; Ospelt et al., 2009). Therefore, targeting FLS might improve clinical outcomes and delay the process of joint destruction in inflammatory arthritis without suppressing systemic immunity.

P-gp is an ATP-dependent drug efflux pump that resides on the cell membrane, and it can actively transfer lipophilic substances out of cells. Those substances not only include steroid hormones and bacterial toxins, but also anti-cancer drugs and some DMARDs. It is therefore speculated that the overexpression of P-gp increases intracellular drug efflux, leading to the depletion of intracellular drug concentration and drug resistance in RRA (van der Heijden et al., 2007).

This hypothesis was further supported by Marchetti et al. (2007), who confirmed that P-gp expression was higher in RA patients with MDR than in healthy controls (Maillefert et al., 1996; Yudoh et al., 1999). In a previous study, we also found that the P-gp activity on lymphocytes in RRA patients was higher than in non-refractory RA patients. Jorgensen et al. (1995) observed that the expression of P-gp mRNA in synovial cells is maintained at high levels in RA patients who are administered a combination of three or more DMARDs. These studies collectively demonstrate that P-gp might be involved in the process of MDR in RRA patients. In the current study, the expression level of P-gp in FLS cells from RRA patients was obviously higher than in their OA counterparts, which supports the hypothesis that P-gp might be associated with the procession of MDR in RRA patients. P-gp can transport a wide range of compounds with different structures and chemical and pharmacological properties, such as steroid hormones and bacterial toxins. This function of transferring hydrophobic substances to the extracellular space can be regarded as the mechanism underlying detoxification by expelling toxic materials. Notably, many drugs for treating RA, such as methotrexate (MTX), glucocorticoids, and gold agents, can be substrates of P-gp (Gottesman and Pastan, 1993). Similarly, our study demonstrated a positive association between P-gp expression and the duration of MTX treatment. Although specimens are scarce owing to the difficulty of harvesting synovial tissues, our data imply that MTX acts as a substrate of P-gp, and the overexpression of P-gp in FLS cells from RRA patients might lead to resistance to MTX.

Previous studies have demonstrated that the expression of P-gp in peripheral blood lymphocytes from RAA patients with drug resistance is significantly higher than in RA patients who are sensitive to DMARDs (Maillefert et al., 1996; Yudoh et al., 1999). Subsequent investigations indicated a positive correlation between P-gp expression in peripheral blood lymphocyte in the RAA group and DAS28 scores as indicators of disease activity. However,

in this study, no relationship was detected between the DAS28 score and P-gp expression in FLS cells from RRA patients, probably resulting from the differential expression of P-gp in FLS cells and lymphocytes. Furthermore, the DAS28 score might vary owing to pre-operative management of RA patients.

In conclusion, high expression of P-gp was observed in FLS cells from RRA patients, which was correlated with longer duration of MTX administration. Our findings imply that the mechanism underlying MDR involves pumping drug molecules out of cells by upregulating the expression of P-gp in synovial cells. Here, we identified for the first time the elevated level of P-gp in FLS cells and its correlation with the progression of rheumatoid inflammation. Therefore, targeting P-gp on FLS cells might prevent and reverse the process of MDR and the occurrence of RRA.

Study limitations

Our study had several limitations that should be acknowledged. First, the sampling size in the present investigation was limited. We intend to accumulate more relevant data and cases in our subsequent study. Second, normal control sampling was not undertaken owing to the scarcity of normal synovial tissues from healthy subjects who are not undergoing knee replacement surgery. Hence, the findings reported here should be validated by more thorough studies.

Conflicts of interest

The authors declare no conflict of interest.

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REFERENCES

Advani R, Visani G, Milligan D, Saba H, et al. (1999). Treatment of poor prognosis AML patients using PSC833 (valspodar) plus mitoxantrone, etoposide, and cytarabine (PSC-MEC). *Adv. Exp. Med. Biol.* 457: 47-56.http://dx.doi.org/10.1007/978-1-4615-4811-9_6

Allaire S, Wolfe F, Niu J and Lavalley MP (2008). Contemporary prevalence and incidence of work disability associated with rheumatoid arthritis in the US. *Arthritis Rheum.* 59: 474-480.http://dx.doi.org/10.1002/art.23538

Arnett FC, Edworthy SM, Bloch DA, McShane DJ, et al. (1988). The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31: 315-324. http://dx.doi.org/10.1002/art.1780310302

Bartok B and Firestein GS (2010). Fibroblast-like synoviocytes: key effector cells in rheumatoid arthritis. *Immunol. Rev.* 233: 233-255. http://dx.doi.org/10.1111/j.0105-2896.2009.00859.x

Beck WT, Grogan TM, Willman CL, Cordon-Cardo C, et al. (1996). Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. *Cancer Res.* 56: 3010-3020.

Brentano F, Kyburz D, Schorr O, Gay R, et al. (2005). The role of Toll-like receptor signalling in the pathogenesis of arthritis. *Cell. Immunol.* 233: 90-96.http://dx.doi.org/10.1016/j.cellimm.2005.04.018

Cordon-Cardo C, O'Brien JP, Casals D, Rittman-Grauer L, et al. (1989). Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* 86: 695-698.http://dx.doi.org/10.1073/pnas.86.2.695

Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, et al. (1990). Expression of the multidrug resistance gene product

(P-glycoprotein) in human normal and tumor tissues. *J. Histochem. Cytochem.* 38: 1277-1287.http://dx.doi.org/10.1177/38.9.1974900

Crofford LJ, Tan B, McCarthy CJ and Hla T (1997). Involvement of nuclear factor kappa B in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. *Arthritis Rheum.* 40: 226-236.http://dx.doi.org/10.1002/art.1780400207

Fojo AT, Ueda K, Slamon DJ, Poplack DG, et al. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proc. Natl. Acad. Sci. USA* 84: 265-269.http://dx.doi.org/10.1073/pnas.84.1.265

Gerlach JH, Endicott JA, Juranka PF, Henderson G, et al. (1986). Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature* 324: 485-489.http://dx.doi.org/10.1038/324485a0

Gottesman MM and Pastan I (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* 62: 385-427.http://dx.doi.org/10.1146/annurev.bi.62.070193.002125

Harris ED, Jr. (1990). Rheumatoid arthritis. Pathophysiology and implications for therapy. *N. Engl. J. Med.* 322: 1277-1289.http://dx.doi.org/10.1056/NEJM199005033221805

Jorgensen C, Sun R, Rossi JF, Costes J, et al. (1995). Expression of a multidrug resistance gene in human rheumatoid synovium. *Rheumatol. Int.* 15: 83-86.http://dx.doi.org/10.1007/BF00262714

Linn SC, Honkoop AH, Hoekman K, van der Valk P, et al. (1996). p53 and P-glycoprotein are often co-expressed and are associated with poor prognosis in breast cancer. *Br. J. Cancer* 74: 63-68.http://dx.doi.org/10.1038/bjc.1996.316

List AF, Kopecky KJ, Willman CL, Head DR, et al. (2002). Cyclosporine inhibition of P-glycoprotein in chronic myeloid leukemia blast phase. *Blood* 100: 1910-1912.

Maillefert JF, Maynadie M, Tebib JG, Aho S, et al. (1996). Expression of the multidrug resistance glycoprotein 170 in the peripheral blood lymphocytes of rheumatoid arthritis patients. The percentage of lymphocytes expressing glycoprotein 170 is increased in patients treated with prednisolone. *Br. J. Rheumatol.* 35: 430-435.http://dx.doi.org/10.1093/rheumatology/35.5.430

Maillefert JF, Duchamp O, Solary E, Genne P, et al. (2000). Effects of cyclosporin at various concentrations on dexamethasone intracellular uptake in multidrug resistant cells. *Ann. Rheum. Dis.* 59: 146-148.http://dx.doi.org/10.1136/ard.59.2.146

Marchetti S, Mazzanti R, Beijnen JH and Schellens JH (2007). Concise review: Clinical relevance of drug drug and herb drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein). *Oncologist* 12: 927-941.http://dx.doi.org/10.1634/theoncologist.12-8-927

Morgan C, Lunt M, Brightwell H, Bradburn P, et al. (2003). Contribution of patient related differences to multidrug resistance in rheumatoid arthritis. *Ann. Rheum. Dis.* 62: 15-19.http://dx.doi.org/10.1136/ard.62.1.15

Ospelt C, Brentano F, Jüngel A, Rengel Y, et al. (2009). Expression, regulation, and signaling of the pattern-recognition receptor nucleotide-binding oligomerization domain 2 in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum.* 60: 355-363.http://dx.doi.org/10.1002/art.24226

Sawai H, Park YW, Roberson J, Imai T, et al. (2005). T cell costimulation by fractalkine-expressing synoviocytes in rheumatoid arthritis. *Arthritis Rheum.* 52: 1392-1401.http://dx.doi.org/10.1002/art.21140

van der Heijden JW, Dijkmans BA, Scheper RJ and Jansen G (2007). Drug Insight: resistance to methotrexate and other disease-modifying antirheumatic drugs--from bench to bedside. *Nat. Clin. Pract. Rheumatol.* 3: 26-34.http://dx.doi.org/10.1038/ncprheum0380

Yudoh K, Matsuno H, Nakazawa F, Yonezawa T, et al. (1999). Increased expression of multidrug resistance of P-glycoprotein on Th1 cells correlates with drug resistance in rheumatoid arthritis. *Arthritis Rheum.* 42: 2014-2015. http://dx.doi.org/10.1002/1529-0131(199909)42:9<2014::AID-ANR32>3.0.CO;2-R