Differential Gene Expression Profiles May Differentiate Responder and Nonresponder Patients with Rheumatoid Arthritis for Methotrexate (MTX) Monotherapy and MTX plus Tumor Necrosis Factor Inhibitor Combined Therapy

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ABSTRACT. ***Objective.*** We aimed to evaluate whether the differential gene expression profiles of patients with rheumatoid arthritis (RA) could distinguish responders from nonresponders to methotrexate (MTX) and, in the case of MTX nonresponders, responsiveness to MTX plus anti-tumor necrosis factor-α (anti-TNF) combined therapy.

Methods. We evaluated 25 patients with RA taking MTX 15–20 mg/week as a monotherapy (8 responders and 17 nonresponders). All MTX nonresponders received infliximab and were reassessed after 20 weeks to evaluate their anti-TNF responsiveness using the European League Against Rheumatism response criteria. A differential gene expression analysis from peripheral blood mononuclear cells was performed in terms of hierarchical gene clustering, and an evaluation of differentially expressed genes was performed using the significance analysis of microarrays program.

Results. Hierarchical gene expression clustering discriminated MTX responders from nonresponders, and MTX plus anti-TNF responders from nonresponders. The evaluation of only highly modulated genes (fold change > 1.3 or < 0.7) yielded 5 induced (4 antiapoptotic and *CCL4*) and 4 repressed (4 proapoptotic) genes in MTX nonresponders compared to responders. In MTX plus anti-TNF nonresponders, the *CCL4*, *CD83*, and *BCL2A1* genes were induced in relation to responders.

Conclusion. Study of the gene expression profiles of RA peripheral blood cells permitted differentiation of responders from nonresponders to MTX and anti-TNF. Several candidate genes in MTX nonresponders (*CCL4*, *HTRA2*, *PRKCD*, *BCL2A1*, *CAV1*, *TNIP1*, *CASP8AP2*, *MXD1*, and *BTG2*) and 3 genes in MTX plus anti-TNF nonresponders (*CCL4*, *CD83*, and *BCL2A1*) were identified for further study. (J Rheumatol First Release July 1 2012; doi:10.3899/jrheum.120092)

Key Indexing Terms:
RHEUMATOID ARTHRITIS MICROARRAY ANALYSIS METHOTREXATE
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Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by chronic and deforming polyarthritis that is accompanied by systemic involvement in many patients. Multiple clinical features and heterogeneous therapy responses may be influenced by genetic, environmental, and immunologic factors[1]. Although some of these features have been used for disease stratification and disease outcome prediction[2,3], they have failed to predict the response to treatment.

Methotrexate (MTX) remains the standard therapy for RA[4], and despite the introduction of other disease-modifying antirheumatic drugs (DMARD), the remission rate for MTX treatment is < 65%[5]. Although pharmacogenomic analyses have been useful in predicting adverse events[6], there are few data about the prediction of the response to MTX. Wessels, *et al*[7] developed a clinical pharmacogenetic model to predict the

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response to MTX in RA patients without any previous treatment. They found that clinical characteristics in association with some polymorphisms of 4 genes involved in the mechanism of action of MTX could predict the response in 60% of the patients with higher or lower disease activity as evaluated by Disease Activity Score (DAS). Although this attempt seems interesting, clinical characteristics [DAS, smoking status, and rheumatoid factor (RF) positivity] still make up the most important portion of the model.

Clinical studies on the efficacy of anti-tumor necrosis factor-α (anti-TNF) agents have shown that about 30% of patients receiving this therapy are nonresponders[3,5]. While many efforts have been made to identify biomarkers for therapy response[6], there is no single clinical or laboratory marker that enables an individual prediction of efficacy for MTX and anti-TNF therapy[8,9,10].

Peripheral blood cells may be suitable for analyzing differential gene expression profiles, providing a framework for selecting clinically relevant biomarkers in RA[11] and distinguishing patients with RA in terms of immunogenetics and treatment features[12]. In this context, transcription signature profiles have been used to predict responses to anti-TNF agents in RA[13,14]; however, there has been no study evaluating differential gene profiles in MTX monotherapy responders and nonresponders. A better understanding of the differential gene profile on the influence of MTX treatment may be helpful in the identification of patients with RA who are most likely to benefit from MTX treatment. We aimed to evaluate whether peripheral blood mononuclear cell (PBMC) differential gene expression profile analysis could distinguish between RA responders and nonresponders to MTX monotherapy. In addition, we also evaluated the transcription profiles of MTX nonresponders who turned to MTX plus anti-TNF therapy, to distinguish further between responders and nonresponders to anti-TNF therapy.

MATERIALS AND METHODS

We evaluated 25 patients who fulfilled the 1987 revised American College of Rheumatology criteria for RA classification[15]. At the beginning of our study, all patients were receiving MTX monotherapy 15–20 mg/week and prednisone 5 mg/day. MTX responders had been receiving it for up to 2 years and MTX nonresponders for 16 weeks. The dose of both drugs was stable for at least 8 weeks before the start of the study and was maintained until the end of it. No patient received a DMARD other than MTX before or during the study. Patients were classified as MTX responders or nonresponders and anti-TNF responders or nonresponders, according to European League Against Rheumatism (EULAR) criteria[16], defining 8 patients as MTX responders and 17 as MTX nonresponders. MTX nonresponders additionally received infliximab intravenously at a dose of 3 mg/kg at 0, 2, 6, and 14 weeks. No patient had received biological treatment before infliximab. These patients were clinically reassessed after 20 weeks of treatment to evaluate the response to anti-TNF agent, according to EULAR criteria[16]. The activity of the disease was measured using the DAS28 (DAS, including a 28-joint count). Blood samples were obtained at the beginning of the study, before the use of anti-TNF therapy.

All patients provided informed consent to participate in the study, which was approved by the local Ethics Committee (protocol 2958/2005). Patients were excluded if they presented other autoimmune or rheumatic diseases, infectious disorders, or positive serology for Chagas disease, hepatitis B or C, or human immunodeficiency virus infection.

Autoantibody analyses. RF was detected by nephelometry, and the test was considered positive at concentrations > 10 IU/ml. Anticitrullinated protein antibody (ACPA) immunoglobulin G (IgG) detection was performed using commercial ELISA kits according to the manufacturer's instructions (Quanta Lite anti-CCP 2; Inova, San Diego, CA, USA) and was considered positive at concentrations > 20 IU/ml.

HLA-DRB1 typing. HLA-DRB1 typing was performed using polymerase chain reaction (PCR)-amplified DNA hybridized with sequence-specific primers using commercial kits (One-Lambda, Canoga Park, CA, USA). DNA was extracted from PBMC obtained after separation on a Ficoll-Hypaque density gradient (Sigma, St. Louis, MO, USA). Individuals carrying certain HLA-DRB1 typing — *01:01, *01:02, *04:01, *04:04, *04:05, *04:08, *10:01, and *14:02 alleles — were considered to pertain to the shared epitope (HLA-SE) group, and they were classified as SE-positive.

RNA extraction and preparation. Twenty milliliters of whole blood was obtained from each individual using Vacutainer-heparin tubes. After separation with Ficoll-Hypaque gradient centrifugation, Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from PBMC, following the manufacturer's instructions. The quality of RNA samples was evaluated using denaturing agarose gel electrophoresis under standard conditions.

Preparation of cDNA microarray. Glass slide cDNA microarrays were prepared on saline-coated Ultra GAPS slides (Corning, Lowell, MA, USA) and were used to evaluate gene expression. The 4500 cDNA sequences were retrieved from the human expressed sequence tag cDNA library (www.lifesciences.sourcebioscience.com). Microarrays were prepared based on published protocols using PCR from the cDNA clones[17] with a Generation III Array Spotter (Amersham Molecular Dynamics, Sunnyvale, CA, USA). The cDNA complex probes derived from patients with RA and from a reference sample were prepared by reverse transcription using 10-μg total RNA and labeled with Cy3 fluorochrome using the CyScribe postlabeling kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Fifteen hours were required for hybridization, followed by washing using an automatic slide processor system (Amersham Biosciences). Microarrays were scanned using a Generation III laser scanner (Amersham Biosciences). Equimolar quantities of cDNA obtained from the total RNA of different human cell strains (Jurkat, Hela, HEp-2, and U343) were used as references for the hybridization. This approach allowed estimation of the relative amount of cDNA target sequences in each microarray spot.

Statistical analyses and clinical and laboratory characteristics of the patients. Student's t test and Fisher's exact test were used to examine group homogeneity. The comparisons between different groups regarding age, time of disease, and DAS28 at baseline and after 20 weeks were performed using a 2-sample t test. Fisher's exact test and contingency tables were used to compare differences between different groups regarding sex, smoking habits, RF, ACPA, and HLA-SE. For all tests, results were considered significant at $p < 0.05$.

Analysis of cDNA microarray data. Microarray image quantification was performed using Spotfinder software (Dana-Farber Cancer Institute, Boston, MA, USA; http://www.tm4.org/). The normalization process was carried out using the R platform, and statistical data were analyzed using Multiexperiment Viewer software (version 3.1; both available from R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org)[18].

The significance analysis of microarrays (SAM) method was used for each independent sample, assigning a score to each gene on the basis of the change in gene expression relative to the SD of repeated measurements. The SAM method uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, i.e., the false discovery rate (FDR = 0.005); its objective is to construct a scatterplot displaying the induced and repressed genes. The program is available at www-stat.stanford.edu/~tibs/SAM/[19]. The data mining of the genes was performed using the SOURCE databases (Genetics Department, Stanford University, Palo Alto, CA, USA; smd-www.stanford.edu/cgi-bin/source/sourceSearch) and DAVID

(National Institute of Allergy and Infectious Diseases, Frederick, MD, USA; david.abcc.ncifcrf.gov). For all comparisons, only genes expressed in at least 80% of the microarray platform were considered analyzed. The microarray experimental plan and data analysis in this study are in accordance with the "minimum information about a microarray experiment" guidelines. The microarray data presented here have been deposited in a public repository, the European Bioinformatics Institute (Wellcome Trust Genome Campus, Cambridge, UK; available from www.ebi.ac.uk/arrayexpress; ArrayExpress accession: E-MEXP-3390).

RESULTS

The clinical and laboratory features of the patients with RA are shown in Table 1. The comparisons of demographic, clinical, and laboratory variables between MTX responders and nonresponders revealed no significant differences (Table 2). Similarly, the comparisons of these variables between responders and nonresponders to anti-TNF agents revealed nonsignificant differences, except for the presence of HLA-SE, which was more frequent in nonresponders to anti-TNF agents ($p = 0.03$; Table 2).

The nonpaired differential gene expression analysis was performed in 2 ways. First, we compared the transcription profile between responders (n = 8) and nonresponders (n = 17) to MTX, and second, we analyzed only the nonresponders to MTX, stratified according to the response or lack of a response to TNF agents. When we compared MTX responders with nonresponders, we observed 535 significant and differentially expressed genes (248 induced and 287 repressed). Overall, modulated genes were primarily implicated in cellular signal transduction, regulation of transcription, protein metabolism, apoptosis regulation, and cell proliferation. The major biological functions of the induced genes included signal transduction (20%), regulation of transcription (12%), cell proliferation (8%), and protein metabolism (8%), whereas the repressed genes were related to signal transduction (16%), regulation of transcription (12%), protein metabolism (11%), and apoptosis (9%), as shown in Figure 1.

Overall, hierarchical clustering of the gene expression profile analysis separated responders from nonresponders to MTX monotherapy (Figure 2). The upper part of the heat map shown in Figure 2 exhibits a cloud of genes that were induced in responders and repressed in nonresponders to MTX, whereas the lower part of the map shows the inverse. To select relevant genes to be discussed, we pinpointed genes that were visually modulated in responders in relation to nonresponders according to the results observed in the heat map. In addition to the qualitative analysis, to restrict the number of highly modulated genes, we selected only the genes for which the magnitude of expression was > 1.3 or < 0.7 (fold change > 1.3 or < 0.7), yielding 9 modulated genes (5 induced and 4 repressed). Among the repressed genes, all were involved in

Table 1. Demographic, clinical, laboratory, and genetic features of patients with rheumatoid arthritis.

Groups	Patient	Age/Sex	Disease Duration, yrs	RF/ ACPA	HLA-DRB1 Allele	HLA-Present	Anti-TNF Agent	DAS28 After MTX Treatment	DAS28 After 20 Weeks	Response to Anti-TNF Agent
MTX responders, n = 8	1	58 M	3	Pos/pos	01:01/04:01	Yes	None	0.14	NA	NA
	2	71 F	6	Pos/pos	13:01/03:01	No	None	2.52	NA	NA
	3	64 M	7	Pos/pos	01:02/03:01	Yes	None	2.05	NA	NA
	4	66 F	2	Pos/neg	03:01/16:01	No	None	1.89	NA	NA
	5	47 F	12	Pos/pos	04:04/04:01	Yes	None	2.37	NA	NA
	6	40 F	2	Pos/pos	04:04/13:01	Yes	None	2.24	NA	NA
	7	37 F	7	Pos/pos	04:01/07:01	Yes	None	2.25	NA	NA
	8	55 F	14	Neg/neg	14:02/16:01	Yes	None	1.54	NA	NA
MTX nonresponders, n = 17	9	47 F	7	Pos/neg	03:01/13:01	No	Infliximab	5.23	2.52	Yes
	10	65 F	11	Pos/pos	01:01/07:01	Yes	Infliximab	7.23	2.05	Yes
	11	56 M	12	Pos/pos	13:01/15:01	No	Infliximab	7.43	3.46	Yes
	12	41 F	1	Pos/pos	03:01/07:01	No	Infliximab	7.96	3.56	Yes
	13	67 M	6	Neg/pos	04:01/11:01	Yes	Infliximab	6.02	3.56	Yes
	14	64 F	1	Neg/pos	11:01/13:01	No	Infliximab	7.24	2.78	Yes
	15	61 F	10	Neg/neg	03:01/11:01	No	Infliximab	5.3	2.43	Yes
	16	59 F	6	Neg/neg	01:01/14:01	Yes	Infliximab	5.97	2.79	Yes
	17	29 F	8	Neg/pos	01:01/14:01	Yes	Infliximab	5.39	2.32	Yes
	18	46 M	2	Pos/pos	04:01/07:01	Yes	Infliximab	6.57	6.2	No
	19	38 F	9	Pos/pos	01:02/03:01	Yes	Infliximab	6.06	5.71	No
	20	39 M	2	Pos/pos	04:01/04:04	Yes	Infliximab	8.76	7.77	No
	21	62 F	3	Pos/pos	01:01/16:01	Yes	Infliximab	6.74	6.88	No
	22	51 F	1	Pos/pos	10:01/12:01	Yes	Infliximab	6.5	5.78	No
	23	53 F	2	Neg/pos	01:02/07:01	Yes	Infliximab	6	5.9	No
	24	55 F	6	Neg/pos	04:01/10:01	Yes	Infliximab	7.03	6.13	No
	25	34 F	3	Pos/pos	04:01/04:01	Yes	Infliximab	4.66	3.55	No

MTX: methotrexate; TNF: tumor necrosis factor; RF: rheumatoid factor; ACPA: anticitrullinated protein antibodies; HLA-SE: shared epitope of HLA-DRB1: DAS28: 28-joint count Disease Activity Score; NA: not applicable.

Table 2. Clinical and laboratory features of patients with rheumatoid arthritis stratified according to their responsiveness [responders (R) or nonresponders (NR)] to methotrexate (MTX) as monotherapy or MTX plus anti-TNF-agent combined therapy.

Features	MTX-R, n = 8	MTX-NR, n = 17	p	MTX-NR + TNF-R, n = 9	MTX-NR + TNF-NR, n = 8	p
Age, yrs, mean (range)	54.7 (37–71)	51 (29–67)	NS[a]	54.3 (29-67)	47.2 (34–62)	NS[a]
Women/men	4/1	3/1	NS[b]	4.5/1	4/1	NS[b]
Disease duration, yrs	6.6	5.4	NS[a]	6.9	3.5	NS[a]
Smoker, %	38	53	NS[b]	44	50	NS[b]
RF+, %	88	65	NS[b]	56	75	NS[b]
ACPA+, %	75	82	NS[b]	67	100	NS[b]
HLA-SE+, %	75.0	70	NS[b]	44	100.0	0.03[b]
DAS28 at baseline, mean ± SD	1.87 ± 0.76	6.47 ± 1.1	< 0.0001[a]	6.42 ± 1.05	6.54 ± 1.1	NS[a]
DAS28 after 20 weeks, mean ± SD	NA	NA		2.83 ± 0.57	5.99 ± 1.20	< 0.0001[a]
MTX, mg/wk, range	15–20	15–20		15–20	15–20	
Prednisone, mg/day	5	5		5	5	

[a] 2-sample t test; [b] Fisher's exact test. MTX-R: responders to methotrexate; MTX-NR: nonresponders to MTX: MTX-NR + TNF-R: nonresponders to MTX but responders to anti-tumor necrosis factor (TNF) agent; MTX-NR + TNF-NR: nonresponders to MTX and nonresponders to anti-TNF agent; RF: rheumatoid factor; ACPA: anticitrullinated protein antibodies; HLA-SE: shared epitope of HLA-DRB1; DAS28: Disease Activity Score including a 28-joint count; NS: not significant; NA: not applicable.

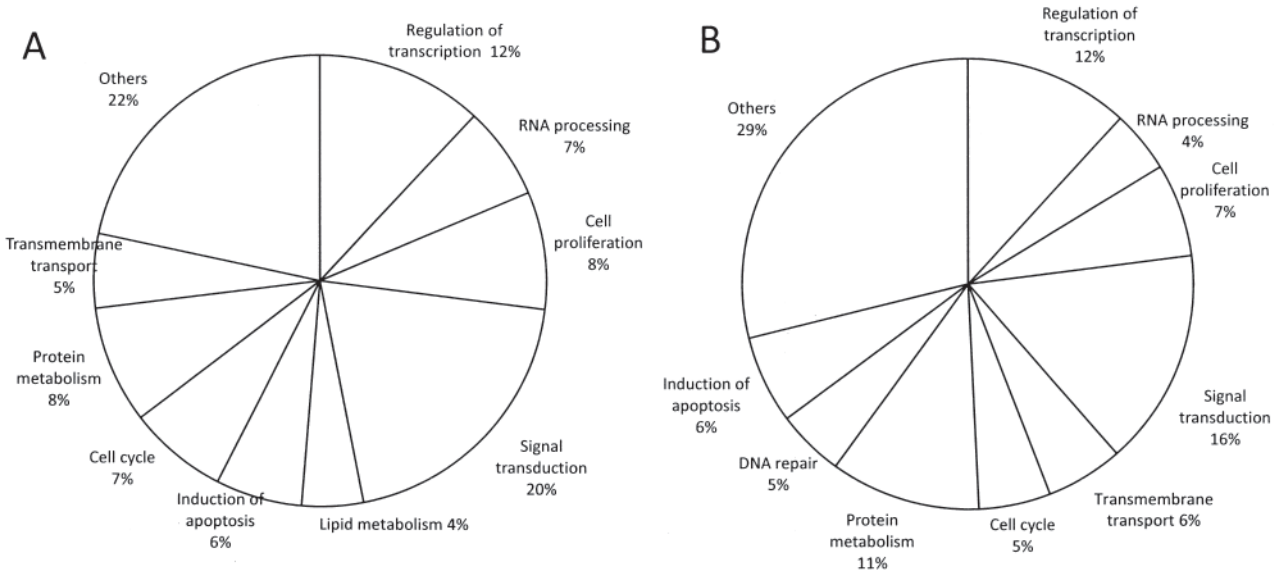

Figure 1. Major biological functions of the 535 modulated genes obtained after comparison between patients with RA who were responders and those who were nonresponders to MTX monotherapy. A. The 238 induced genes. B. The 287 repressed genes.

proapoptotic processes, including *protein kinase DNA-activated catalytic polypeptide* (*PRKDC*), *caspase 8 associated protein 2* (*CASP8AP2*), *caveolin 1* (*CAV1*), and *HtrA serine peptidase 2* (*HTRA2*). Four out of 5 induced genes were involved in antiapoptotic processes, including *BCL2-related protein A1* (*BCL2A1*), *BTG family member 2* (*BTG2*), *TNFAIP3 interacting protein 1* (*TNIP1*), and *MAX dimerization protein 1* (*MXD1*). In addition, we observed 1 induced gene involved in immune response and inflammation [*chemokine (C-C motif) ligand 4* (*CCL4*); Table 3].

All patients who failed to respond to MTX (n = 17) additionally received infliximab, and after 20 weeks of treatment, 9 were classified as responders and 8 were classified as nonresponders to the anti-TNF agent (Table 1). The comparisons of the transcription profiles of responders to nonresponders to anti-TNF agents disclosed 288 differentially expressed genes (192 induced and 96 repressed), which were implicated in signal transduction, regulation of transcription, cell cycle, protein metabolism, and apoptosis regulation. The biological functions of the induced genes included signal transduction (14%), regulation of transcription (13%), protein metabolism (11%), cell cycle (9%), and apoptosis (9%), whereas the repressed genes were related to the regulation of transcription (13%), signal transduction (12%), cell cycle (11%), protein metabolism (9%), and apoptosis (9%; Figure 3).

The second hierarchical clustering analysis performed

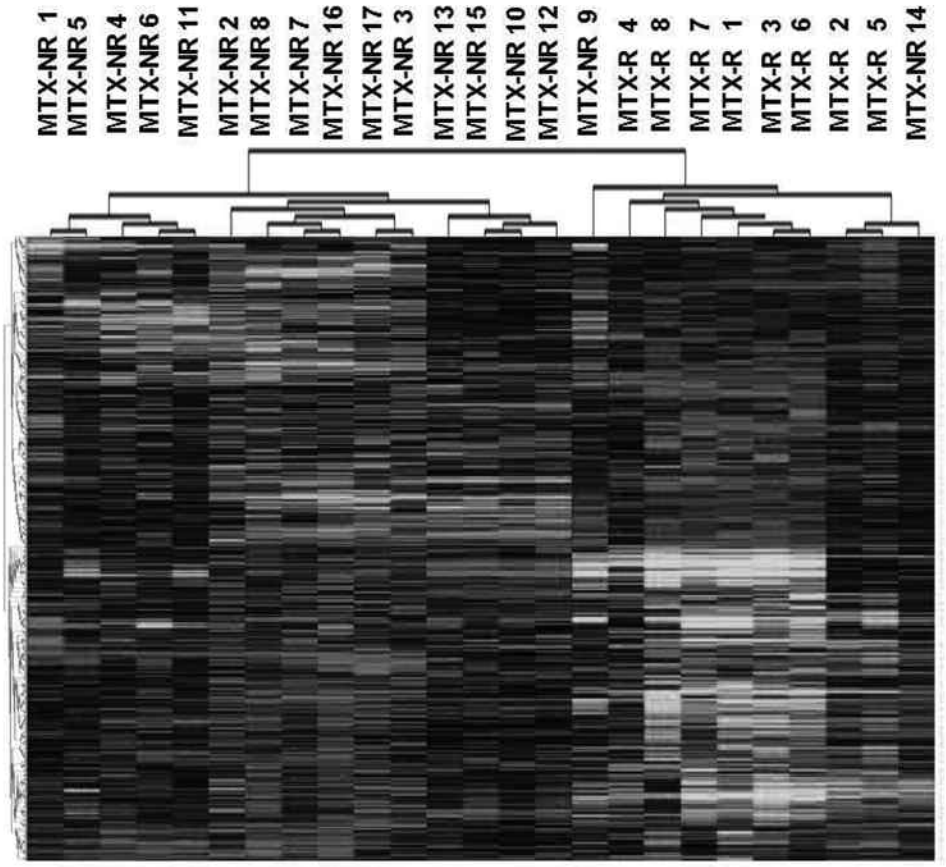

Figure 2. Hierarchical clustering of differential gene expression in patients with RA, stratified according to the response to MTX therapy (MTX-R: MTX responders; MTX-NR: MTX nonresponders). The dendrogram over the heat map shows distinct hybridization profiles for MTX responders and nonresponders.

Table 3. Highly modulated genes (0.7 < fold change > 1.3) observed in patients with RA after comparison between responders (n = 8) and nonresponders (n = 17) to methotrexate (MTX) monotherapy.

Symbol	Gene	Process	Fold Change (MTX-NR vs MTX-R)
Apoptosis			
HTRA2	*HtrA serine peptidase 2*	Proapoptotic	0.69
CAVI	*Caveolin 1*	Proapaptotic (cell death TNF-induced)	0.65
CASP8AP2	*Caspase 8 associated protein 2*	Proapoptotic	0.54
PRKDC	*Protein kinase, DNA-activated, catalytic polypeptide*	Proapoptotic	0.49
BCL2A1	*BCL2-related protein A1*	Antiapoptotic	1.62
MXD1	*MAX dimerization protein 1*	Antiapoptotic	1.43
TNIP1	*TNFAIP3 interacting protein 1*	Antiapoptotic TNF-dependent	1.35
BTG2	*BTG family, member 2*	Antiapoptotic	1.32
Immune response			
CCL4	*Chemokine (C-C motif) ligand 4*	Inflammatory response	1.89

MTX-NR: nonresponders to methotrexate; MTX-R: responders to MTX; TNF: tumor necrosis factor.

among responders and nonresponders to the anti-TNF agents clearly grouped these patients in separate clusters, as shown in Figure 4. The visual analysis of the heat map, also shown in Figure 4, shows a cluster of induced genes (upper part of the figure) and a cluster of repressed genes (lower part of the figure) in the nonresponder group. Regarding the profile of the responder group, a cloud of repressed genes can be observed in the middle of Figure 4, and another cloud of induced genes appears at the bottom. The quantitative analysis, restricted to genes for which the expression was > 1.3 or < 0.7 (fold change

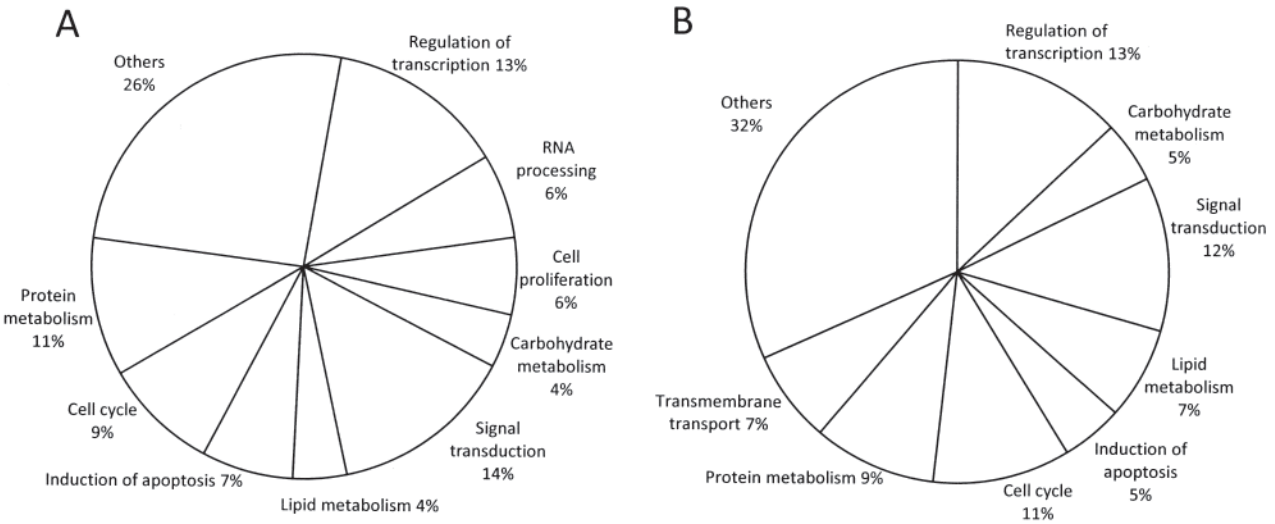

Figure 3. Major biological functions of the 288 modulated genes obtained after comparison between patients with RA who were responders and those who were nonresponders to MTX plus anti-TNF agent combined therapy. A. The 192 induced genes. B. The 96 repressed genes.

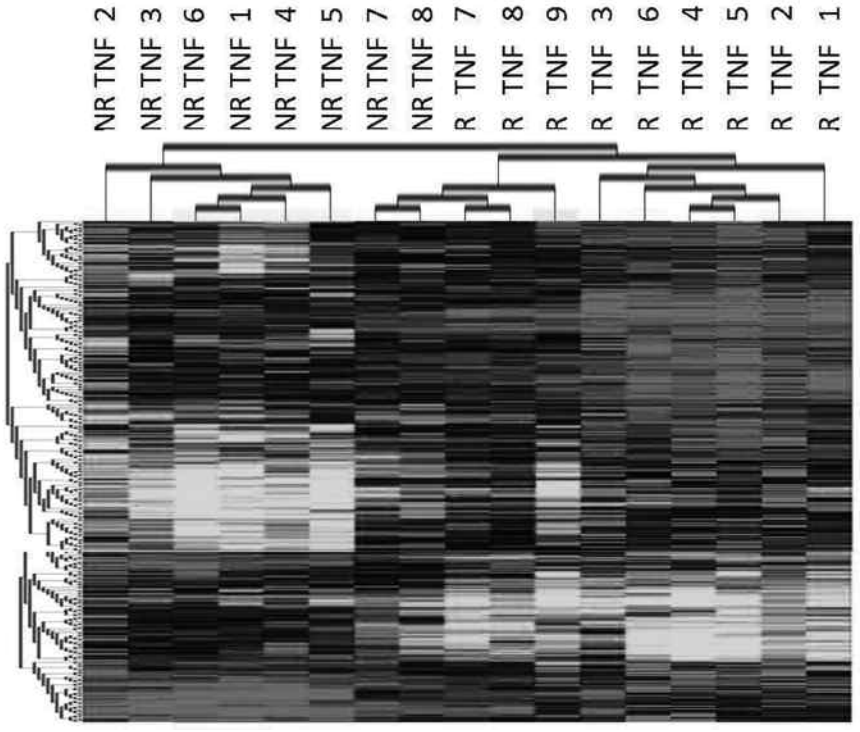

Figure 4. Hierarchical clustering of differential gene expression in patients with RA stratified according to response to MTX plus anti-TNF agent combined therapy (R TNF: MTX + anti-TNF agent responders; NR TNF: MTX + anti-TNF agent nonresponders). The dendrogram over the heat map shows distinct hybridization profiles for MTX + anti-TNF agent responders and nonresponders.

> 1.3 or < 0.7), yielded 3 modulated genes; all of these were repressed. One gene was related to the inhibition of apoptosis (*BCL2A1*) and the other 2 were related to immune and inflammatory responses [*CCL4* and *CD83 molecule* (*CD83*); Table 4]. The *BCLA2A1* and *CCL4* genes were induced in nonresponders to MTX and in nonresponders to anti-TNF agents, as shown in Tables 3 and 4.

DISCUSSION

The evaluation of differential gene expression profiles has

Table 4. Highly modulated genes (0.7 < fold change > 1.3) observed in patients with RA after comparison between responders (n = 9) and nonresponders (n = 8) to methotrexate plus anti-tumor necrosis factor (TNF) agent combined therapy.

Symbol	Gene	Process	Fold Change (TNF-R vs TNF-NR)
Apoptosis			
BCL2A1	*BCL2-related protein A1*	Inhibition of apoptosis	0.57
Immune response			
CCL4	*Chemokine (C-C motif) ligand 4*	Inflammatory response	0.33
CD83	*CD83 molecule*	Humoral response	0.67

TNF-R: responders to anti-TNF therapy; TNF-NR: nonresponders to anti-TNF therapy.

been useful as a tool to discriminate between hybridization signatures in tissue specimens and in PBMC, which have been used as reporters of subjacent autoimmune or chronic inflammatory disease[20]. Although tissue and cells obtained from the site of inflammation may more accurately reflect the pathogenic features of a disorder, sample accessibility is crucial. In this regard, peripheral immune cells are key sentinels of host defense, being used to identify novel disease biomarkers and treatment response[21,22,23].

Because the presence of HLA-SE, RF, and ACPA[11,24,25] and coadjuvant RA treatment (particularly with corticosteroids[26,27]), may influence transcription profiles in patients with RA, it is notable that all these variables were very similar between MTX responders and nonresponders. In addition, the dose of MTX and the type of anti-TNF agents given was strictly controlled in all patients. The control of demographic, clinical, laboratory, and treatment features contributed to a better comparison between responders and nonresponders, permitting a more accurate interpretation of the results.

There is little information regarding the transcription profiles of patients with RA who respond or do not respond to MTX treatment. The only study available in the literature evaluated the effect of *in vitro* MTX treatment on RA and on control synovial fibroblasts, reporting that MTX reversed the modulated expression of genes related to apoptosis and cell adhesion in RA fibroblasts to the levels observed in control fibroblasts[28]. In our series, despite the small number of patients, we clearly observed modulation of genes implicated in apoptosis in MTX nonresponder patients when compared to responders. Of these genes, 4 were repressed and associated with proapoptotic mechanisms (*HTRA2*, *CAV1*, *CASP8AP2*, and *PRKDC*), and 4 were induced and associated with antiapoptotic processes (*BCL2A1*, *MXD1*, *TNIP1*, and *BTG2*). It is interesting to observe that 2 of the genes modulated in MTX nonresponders, 1 proapoptotic (*CAV1*) and 1 antiapoptotic (*TNIP1*), are related to TNF-dependent apoptosis. In addition, *CAV1* was also induced in responders to anti-TNF agents (fold change 1.23), suggesting that a balance between pro- and antiapoptotic genes may contribute to MTX response. Indeed, MTX reduces cell viability, and this effect may be correlated with the induction of apoptosis, especially in synovial cells, T cells, and monocytes from patients with RA[29,30]; these effects should be expected in MTX responders. In addition to apoptosis, MTX may also suppress T cell activation, mediated in part by adenosine[31]. Thus, the lack of response to MTX in patients with RA may be due to a lack of apoptosis induction and impairment of T cell suppression, as suggested by Brinker and Ranganathan[6].

Considering the transcription profiles of MTX nonresponders who received anti-TNF agents (infliximab), various approaches have been developed to identify a transcript signature associated with a response to anti-TNF agents in patients with RA[13,32]; however, the results have been heterogeneous because of several variables, including a lack of homogeneity of the patients in terms of clinical and laboratory data and the use of coadjuvant therapies[13,14]. Although almost all of these variables were controlled in the present series, HLA-SE was present in all nonresponders and in only 44% of the responders to anti-TNF therapy. HLA-SE is strongly associated with susceptibility and severity of disease in patients with RA[33,34]; however, it has not been identified as a predictive marker for anti-TNF response[35,36]. It is emphasized that the major focus of our study was evaluation of the gene profiles associated with MTX therapy, and blood collection was performed before anti-TNF therapy was administered, i.e., the response to that therapy was evaluated 20 weeks afterward. This approach permitted evaluation of the transcription profiles before administration of the anti-TNF agent (Figure 4). In this context, the *BCL2A1* gene, involved in the inhibition of apoptosis, was repressed in anti-TNF agent responders compared with nonresponders (Table 4). In the first analysis, patients who did not respond to MTX monotherapy presented induction of the *BCL2A1* gene (fold change 1.62) compared with MTX responders (Table 3). This result indicated that MTX nonresponders, exhibiting downregulation of the *BCL2A1* gene, responded to anti-TNF therapy. This effect could be an additional mechanism of therapeutic response induced by anti-TNF agents. Because Bcl-2 inhibitors (ABT-737) are small molecules with apoptotic

activity, potentially useful in cancer therapy[37], these agents could be potential drugs for RA therapy.

The *CCL4* and *CD83* genes were also downregulated in responders to anti-TNF agents compared with nonresponders (Table 4). CCL4 (MIP1-ß) is a potent chemoattractant to T cells and natural killer cells, antigen-presenting cells, and monocytes, and it appears to have an important proinflammatory role in RA[38]. Given that patients with RA present with elevated CCL4 levels[39] and that CCL4 is upregulated in MTX and MTX plus anti-TNF agent nonresponders, this chemokine may be further considered as a potential target for these patients. CD83 is a surface marker of dendritic cell maturation, and it has been shown to take part in the activation of T cells and B cells[40,41]. Given that a T cell activation molecule is upregulated in nonresponders to MTX and anti-TNF agents, this molecule may be a potential marker for nonresponders, meriting further studies. These results suggest that nonresponders to MTX plus anti-TNF agents exhibit a proinflammatory profile, characterized by increased lymphomononuclear chemoattractants (*CCL4*), activated lymphocytes, and antigen-presenting cells (*CD83*), and that nonresponders to MTX monotherapy exhibit increased expression of antiapoptotic genes (*BCL2A1*, *MXD1*, *TNIP1*, *and BTG2*) and decreased expression of proapoptotic genes (*HTRA2*, *CAV1*, *CASP8AP2*, and *PRKDC*) in addition to the increase in *CCL4* expression. Conversely, in a similar study evaluating the transcription profiles of PBMC of responders and nonresponders to anti-TNF agents, Juliá, *et al* reported 8 genes that could be used as models for predicting the response to anti-TNF agents; 3 of them (*GNLY*, *SLC2A3*, and *MXD4*) are associated with apoptosis mechanisms[42].

Our work confirms growing knowledge about apoptosis as a key mechanism in the response to anti-TNF agents, driven by the modulation of proapoptotic Bcl-2 family and caspase activity, as recently reviewed[43].

One could argue that use of anti-TNF agents is restricted to infliximab. We chose only 1 drug and only 1 mechanism of action to ensure that the findings in our study could not be attributed to other anti-TNF agent. The gene expression profile of patients using etanercept has already been assessed. Koczan, *et al*, evaluating 19 patients with RA in a synthetic DMARD and corticosteroid uncontrolled study, described the cDNA microarray as a useful tool to predict clinical response to etanercept after 3 months of treatment[13] using an 8-gene model, including *CCL4*. The cDNA microarray showed 42 differentially expressed genes between responders and nonresponders to etanercept. Similar to our results, these genes were found to be involved in regulation of transcription, signal transduction, immune response, metabolism, and protein binding and transport. Thus, we suppose that the modulated genes can vary when different anti-TNF agents are used, but the biological processes involved seem to be similar.

With our study of the gene expression profiles of RA PBMC, it was possible to distinguish responders from nonresponders to MTX and responders and nonresponders to MTX plus anti-TNF agents. We also found candidate genes relating to mechanisms of disease and treatment possibilities that should be studied further.

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