

# Association between the response to B cell depletion therapy and the allele\*2 of the HS1,2A enhancer in seropositive rheumatoid arthritis patients

S. Canestri<sup>1</sup>, M.C. Totaro<sup>1</sup>, E. Serone<sup>2</sup>, B. Tolusso<sup>1</sup>,  
D. Frezza<sup>2</sup>, E. Gremese<sup>1</sup>, G. Ferraccioli<sup>1</sup>

<sup>1</sup>Division of Rheumatology, Institute of Rheumatology and Affine Sciences,  
Catholic University of Sacred Heart, Rome;

<sup>2</sup>Laboratory of Genetics, Department of Biology "Enrico Caffè", University of Rome "Tor Vergata", Rome

Work awarded  
at the XLVIII Congress  
of the Italian Society  
of Rheumatology (SIR),  
Rimini 2011

## SUMMARY

**Objective.** Several studies underline the relevance of the genetic background for the response to therapy. We evaluated the relationship between the polymorphism of the HS1,2A enhancer, located in the 3' regulatory region of the heavy immunoglobulin chain (IgH), and the response to B cell depletion therapy (BCDT) with Rituximab (RTX).

**Methods.** Fifty rheumatoid arthritis (RA) patients (42 women; disease duration 13.9±10.6 years) treated with RTX, not responsive to previous DMARDs and/or TNF $\alpha$  inhibitors therapies, and 220 healthy subjects were enrolled in the study. Patients were genotyped for HS1,2A enhancer polymorphism, as previously described. Disease activity was assessed every three months according to the European League Against Rheumatism's (EULAR) criteria.

**Results.** All RA patients were seropositive for at least one of the tested autoantibodies: rheumatoid factor (FR IgA, FR IgM e FR IgG), anti-cyclic citrullinated peptides (anti-CCP IgA, anti-CCP IgM e anti-CCP IgG) and anti-vimentin antibodies. RA patients had an increased frequency of the allele\*2 (60.0%) of the HS1,2A enhancer compared to healthy subjects (42.0%; OR(95%ICs): 2.07 (1.33-3.22)). Patients with a good EULAR response at 6 months follow-up visit had an increased frequency of genotype 2/2 (47.1%) compared to poor-responders RA patients (genotype 2/2: 18.2%, OR(95%ICs): 4.00 (1.09-14.68)). All the patients with a good EULAR response had the allele\*2, thus showing a possible association with the allele in this population.

**Conclusions.** The presence of allele\*2 seems to be related to a good response to BCDT with RTX in seropositive RA patients, thus highlighting the role of the HS1,2A enhancer in B cell maturation and class-switch recombination.

**Key words:** HS1,2A, rheumatoid arthritis, Rituximab, SNP, B lymphocytes.

Reumatismo, 2012; 64 (6): 368-373

## ■ INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by inflammation of the joint synovial tissue. The early phases of the disease have a great importance and are crucial for therapeutic intervention (1-3). It has been widely demonstrated, both clinically and biologically, that RA is a multifactorial disease, driven by different processes involved in the regulation of autoimmune homeostasis.

Recently, B lymphocytes showed a central

role in the pathogenesis of RA, due to the numerous tasks they accomplish in a normal immune response. B cells are mainly known for their role as effectors of humoral immunity through the secretion of antibodies. Moreover, they are able to start the immune response as "antigen presenting cells" together with other cell lines (such as dendritic cells and macrophages). Finally, B cells secrete interleukins and other factors, thus promoting the progression of inflammation, the cell recruitment and the architectural organization of lymphatic tissue (4).

Corresponding author:  
Gianfranco Ferraccioli, MD  
Division of Rheumatology, Institute of  
Rheumatology  
and Affine Sciences, Catholic University of  
Sacred Heart-Rome  
Via G. Moscati, 31 - 00168 Rome, Italy  
E-mail: gf.ferraccioli@rm.unicatt.it

Considering B lymphocytes as a therapeutic target, in recent years highly selective immunologic therapies have been developed. Rituximab (RTX), a chimeric anti-CD20 monoclonal antibody, has recently been introduced for the treatment of RA patients not responding to DMARDs therapies and/or biologic drugs. RTX depletes B cells expressing CD20 antigen, but not the pre-B cells or plasma cells (5,6). The depletion of CD20+ B cells in RA patients is complete after one month from the first treatment and is sustained for several months (7). This drug acts through various mechanisms such as apoptosis, antibody dependent cellular cytotoxicity and complement activation (8). Though B cells depletion is effective in the majority of patients, a percentage of them do not respond to the treatment.

To date, it has been demonstrated that the positivity of rheumatoid factor (RF) and of anti-citrulline antibodies (ACPA) is associated with a better clinical response, while the previous use of one or more anti-TNF drugs has been shown to be related with a worse clinical response (9-11).

With the introduction of new classes of biologic drugs, the identification of biomarkers of response to therapy certainly became one of the most important goals.

Several studies underline the importance of the genetic background for the individual susceptibility to autoimmune diseases such as RA, but only recently the attention switched to the research of possible genetic markers which may be able to predict the response to different therapies, the BCDT included (12).

A polymorphism of the HS1.2A enhancer located in the 3' regulatory region of the locus for the immunoglobulin heavy chain (IgH) (13) has been associated with susceptibility to RA, systemic lupus erythematosus, systemic sclerosis, psoriasis, psoriatic arthritis and celiac disease (14-18).

The purpose of this study was to evaluate the frequency of the alleles of the HS1.2A enhancer, and in particular of the allele\*2, in a cohort of RA patients treated with RTX who presented positivity for at least one of the tested autoantibodies: IgM, IgA

and IgG isotypes of RF and of ACPA, and anti-vimentin antibodies and to determine whether the presence of the allele\*2 was associated with a different response to BCDT.

## ■ MATERIALS AND METHODS

### *Patients*

We enrolled 50 patients with RA (1987 American College of Radiology's criteria), seropositive for at least one autoantibody and not responsive to the conventional therapy with DMARDs and/or anti-TNF. These patients were treated with RTX in the Division of Rheumatology at the Catholic University of the Sacred Heart of Rome. We enrolled also 220 healthy subjects, matched for age and sex, as a control group.

### *Therapy*

All patients were treated with RTX with a basal scheme of 2 infusions (1 g or 500 mg at days 0 and 15). Further treatments were performed with a scheme of two infusions (500 mg x 2) or hematologic scheme (375 mg/m<sup>2</sup> on days 1, 7, 14, 21), at least six months from baseline.

### *Evaluation of the response to therapy*

After 6 months from baseline, patients were divided into three groups, according to EULAR criteria: good responders (DAS<2.4 together with a decrease of at least 1.2 from baseline), poor responders (DAS<2.4) and patients in remission (DAS<1.6) (19).

*Laboratory analyses.* Erythrocyte sedimentation rate (ESR), levels of C-reactive protein (CRP) and immunoglobulins were determined after 6 months from the therapy initiation for each patient. Moreover every patient was tested for plasma levels of rheumatoid factor (RF IgA, RF IgM and RF IgG), anti-citrulline antibodies (IgA, IgM and IgG ACPA) and anti-vimentin antibodies (anti-MCV) at baseline and every six months, using ELISA kit (Phadia, Freiburg, Germany for anti-CCP; Orgentec Diagnostika GmbH, Mainz, Germany for anti-vimentin antibodies and rheumatoid

factors). The following values were considered positive: IgG ACPA  $\geq 7.0$  U/mL, IgM ACPA  $\geq 100$  U/mL, IgA ACPA  $\geq 2.2$  U/mL, IgG RF  $\geq 20$  U/mL, IgM RF  $\geq 20$  U/mL, IgA RF  $\geq 20$  U/mL, anti-MCV  $\geq 20$  U/mL.

At baseline and after 6 months, plasma levels of IL-6 and BAFF were measured by ELISA (R & D Systems, Abingdon, UK). The test sensitivity was 2.2 pg/mL for the IL-6 assay and 3.8 pg/mL for the BAFF assay.

#### **DNA sample collection and HS1,2A genotyping**

DNA extraction of patients and control subjects was performed with Quickgene DNA Whole Blood kit (Life Science, Tokyo, Japan).

The selective PCR of the HS1,2A enhancer polymorphism situated in the 3' regulatory region of the immunoglobulin heavy chain (IgH) was performed as described from Giambra et al. (20).

#### **Statistics**

Data were analyzed with SPSS 20.0 (SPSS, Chicago, IL, USA) and Graph-Pad 5.0 (San Diego, CA, USA). Categorical and quantitative variables were respectively described as numbers, percentages (%) and mean  $\pm$  standard deviation (SD). Mann-Whitney's test was used to compare continuous variables. Categorical variables were analyzed using the  $\chi^2$  test or Fisher's exact test, according to the sample size. Odds ratios (OR) with 95% confidence intervals (CIs 95%) were calculated. A value of  $P < 0.05$  was considered statistically significant.

## ■ RESULTS

#### **Baseline demographic, clinical and immunological characteristics of patients**

Clinical data of the 50 RA patients enrolled in this study are shown in Table I. At baseline, patients showed high disease activity (DAS:  $4.1 \pm 1.4$ ) and high disability (HAQ:  $1.6 \pm 0.9$ ). The mean disease duration at baseline was  $13.9 \pm 10.6$  years. All patients were seropositive for at least one of the tested autoantibodies. Sixty four per cent

**Table I** - Demographic, clinical and laboratory data of rheumatoid arthritis patients at baseline.

	RA patients N=50
Sex: women, n (%)	42 (84.0)
Age, mean $\pm$ SD (year)	$56.5 \pm 15.5$
Disease duration, mean $\pm$ SD (year)	$13.9 \pm 10.6$
CRP (mg/L), mean $\pm$ SD	$19.3 \pm 19.0$
ESR (mm/1 <sup>h</sup> hour), mean $\pm$ SD	$44.0 \pm 25.0$
DAS, mean $\pm$ SD	$4.2 \pm 1.3$
HAQ, mean $\pm$ SD	$1.6 \pm 0.9$
ACPA IgG $\geq 7.0$ U/mL, n (%)	28/35 (80.0)
ACPA IgM $\geq 100$ U/mL, n (%)	8/35 (22.9)
ACPA IgA $\geq 2.2$ U/mL, n (%)	19/35 (54.3)
RF-IgG $\geq 20$ U/mL, n (%)	35/35 (100.0)
RF-IgM $\geq 20$ U/mL, n (%)	28/50 (56.0)
RF-IgA $\geq 20$ U/mL, n (%)	16/50 (32.0)
Anti-MCV $\geq 20$ U/mL, n (%)	44/50 (88.0)

RA, rheumatoid arthritis; SD, standard deviation; CRP, C reactive protein; ESR, erythrocyte sedimentation rate; DAS, disease activity score; HAQ, health assessment questionnaire; ACPA, anti-citrulline autoantibodies; RF, rheumatoid factor; anti-MCV, anti vimentin.

of patients were treated with Methotrexate. Thirty two per cent of patients were naïve to TNF $\alpha$  inhibitors therapy, while the remaining received one or more therapeutic attempts with anti-TNF $\alpha$ .

#### **Distribution of genotypes of the HS1,2A enhancer at baseline**

The distribution of genotypes and alleles of the HS1,2A is shown in Table II. The allele\*2 was more frequent in RA patients (60.0%) compared to controls (42.0%, OR (95% CI): 2.07 (1.33 to 3.22)). On the contrary, the allele\*1 was more prevalent in controls (RA: 23.0%, controls: 45.0%, OR (95% CI): 0.36 (0.22-0.60)). The evaluation of each genotype showed a higher frequency of genotype 2/2 of the HS1,2A in patients with RA (28.0%) compared to controls (17.3%, OR (95% CI): 1.86 (0.92 -3.79)). No association arose between the presence of the allele\*2 and the autoantibody positivity (anti-MCV, ACPA and RF) at baseline, and inflammatory markers (ESR, CRP) at baseline. Furthermore, the allele\*2 was not related with disease activity (DAS), neither with disability (HAQ) at baseline.

**Table II** - Allelic distribution of HS1,2A enhancer in rheumatoid arthritis patients.

Genotype	RA patients N=50		Control subjects N=220		OR (CI 95%)
	N	%	N	%	
1/1	2	4.0	50	22.7	0.14 (0.03-0.60)
2/2	14	28.0	38	17.3	1.86 (0.92-3.79)
3/3	-	-	1	0.5	-
4/4	-	-	2	1.0	-
1/2	17	34.0	78	35.5	0.94 (0.49-1.79)
1/3	-	-	9	4.1	-
1/4	2	4.0	11	5.0	0.79 (0.17-3.69)
2/3	-	-	9	4.1	-
2/4	15	30.0	22	10.0	3.86 (1.83-8.15)
3/4	-	-	-	-	-
<b>Allele</b>					
*1	0.230±0.042		0.450±0.024		0.36 (0.22-0.60)
*2	0.600±0.049		0.420±0.024		2.07 (1.33-3.22)
*3	-		0.045±0.099		-
*4	0.170±0.038		0.084±0.013		2.23 (1.20-4.15)

RA, rheumatoid arthritis; OR, odds ratio (compared to controls); CI, confidence intervals.

### ***HS1,2A enhancer and response to BCDT***

Patients were clinically evaluated after 6 months of RTX therapy. The 34.0% of RA patients reached a “good EULAR response” at the 6<sup>th</sup> month of follow-up after RTX treatment.

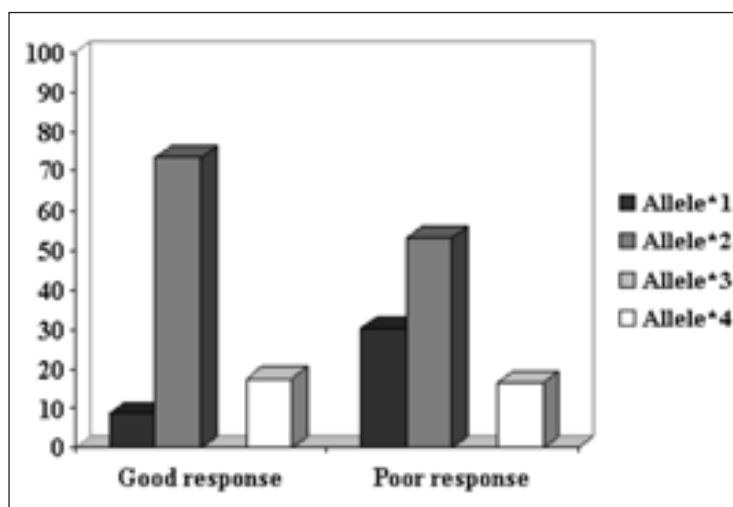
Genotype 2/2 was more prevalent (47.1%) in RA patients with a good clinical response at the 6<sup>th</sup> month of follow-up compared to poor responders (18.2%, OR (IC 95%): 4.00 (1.09-14.68)). Concerning the distribution of the single alleles, all patients with DAS<2.4 were carriers of the allele\*2, thus confirming an association with this allele in this population (Figure 1).

Considering plasma levels of IL-6 and BAFF, only patients carrying the allele\*2 showed significantly increased plasma levels of BAFF at 6<sup>th</sup> month of follow-up after RTX treatment (T0 vs T6: 722.7±604.0 pg/mL vs 2545.0±1820.0 pg/mL, P<0.001) and significant decreased plasma levels of IL-6 (T0 vs T6: 28.66±57.10 pg / mL vs 11.14±27.05 pg / mL, P=0.05). There were no significant changes in plasma levels of IL-6 and BAFF in non-carriers of the allele\*2.

## ■ DISCUSSION

An important goal in RA disease is the op-

timization of therapeutic interventions and the prediction of their effectiveness. While conventional DMARDs have shown good clinical efficacy (21, 22), the modern biological therapies allowed a significant improvement on short and long-term prognosis in RA patients. The lack of direct comparison studies between the various biological therapies do not allow the development of a specific treatment algorithm (23).



**Figure 1** - Allelic distribution of HS1,2A enhancer according to clinical response at the 6th month of follow-up (EULAR criteria) in RA patients treated with RTX.

BCDT with RTX is one of the treatments used in patients with seropositive and erosive RA not responding to anti-TNF. Various studies suggest the importance of using BCDT after therapeutic failure of an anti-TNF drug (24, 25).

Several studies focused their attention to the research of possible genetic markers that can predict a different response to BCDT, such as the IL-6 (IL-6-174C/G) and the Fc receptor (FcγRIII) polymorphisms (26, 27).

Recently, a polymorphism of the HS1,2A enhancer located in the 3' regulatory region of the locus for the immunoglobulin heavy chain (IgH) has been associated with susceptibility to various autoimmune diseases, including RA (14-18). The frequency of the allele\*2 of the HS1,2A enhancer was significantly higher in RA patients compared with control subjects.

In the present study we investigated the distribution of allelic polymorphism of the HS1,2A enhancer in a cohort of patients with seropositive RA treated with RTX. The analysis showed a significant increase of the allele\*2 in patients with RA compared to controls. The allele\*1 was rather more present in controls, thus validating the association of the allele\*2 with RA susceptibility (18).

The analysis of the response to therapy with RTX also demonstrated an increased percentage of genotype 2/2 and allele\*2 in patients with RA who obtained a "good EULAR response" after 6 months of therapy compared to poor responder patients.

The analysis of changes in the cytokine pattern gave a further confirmation. Only in patients carrying the allele\*2 there was a significant reduction of IL-6 plasma levels and increased levels of BAFF plasma levels at 6<sup>th</sup> month of follow-up.

Our data seem to show that the allele\*2 of the HS1,2A enhancer is associated with a good clinical response in patients with a seropositive RA treated with RTX. A possible interpretation of these results may be given by the recent demonstration, by EMSA method, of the presence of a binding site for NF-κB in the allele\*2 (absent in the allele\*1) (28). The NF-κB is an impor-

tant transcription factor that regulates the general inflammatory response.

B cells of patients carrying the allele\*2 may therefore have a greater role in the pathogenesis of the disease, compared to lymphocytes from patients without this allele, due to the possibility of being more active in the inflammatory response. Therefore, BCDT may be more effective in patients carrying the allele\*2.

The ability to predict the therapeutic response through the use of biomarkers, represents one of the biggest challenges in the upcoming years.

Since the allele\*2 of HS1,2A is a genetic marker of RA, the association with the response to B cells depletion therapy adds a further confirmation to the molecular link between RA and B lymphocytes and their key role in the pathogenesis of autoimmune diseases.

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