Anti-inflammatory genes associated with multiple sclerosis: A gene expression study

From SNPs association to RNA expression: novel anti-inflammatory genes down-regulated in Multiple Sclerosis

Perga S.[1 2], Montarolo F.[1 2]*, Martire S.[1 2]*, Berchialla P.[3], Malucchi S.[2], Bertolotto A.[1 2]

Affiliation

[1] Neuroscience Institute Cavalieri Ottolenghi (NICO), Orbassano (TO) Italy

[2] University Hospital San Luigi Neurobiology Unit, Neurologia 2 (CReSM - Regional Referring Center of Multiple Sclerosis), Orbassano (TO) Italy

[3] Department of Clinical and Biological Sciences, University of Turin, Torino (TO), Italy

* these authors equally contributed to this work

Corresponding author at: Simona Perga, Neuroscience Institute Cavalieri Ottolenghi (NICO) - University Hospital San Luigi Neurobiology Unit, Neurologia 2 (CReSM - Regional Referring Center of Multiple Sclerosis), Orbassano (TO) Italy. Tel.: +39 011 670 6600

E-mail address: simona.perga@unito.it

Abstract

Multiple sclerosis (MS) is an autoimmune inflammatory disease of the central nervous system caused by a complex interaction between multiple genes and environmental factors.

HLA region is the strongest susceptibility locus, but recent huge genome-wide association studies identified new susceptibility genes. Among these, BACH2, PTGER4, RGS1 and ZFL36L1 were highlighted. Here, a gene expression analysis revealed that three of them, namely BACH2, PTGER4 and ZFL36L1, are down-regulated in MS patients' blood cells compare to healthy subjects. Interestingly, all these genes are involved in the immune system regulation with predominant anti-inflammatory role and their reduction could predispose to MS development.

Key words: Multiple Sclerosis, inflammation, gene expression, GWAS

1. Introduction

Multiple sclerosis (MS) is a complex autoimmune inflammatory disease of the central nervous system in which environmental and genetic factors converge with epigenetic and post-genomic regulatory events.

The leading role of genetic factors is supported by several studies of MS families (Robertson et al., 1996 ; Ebers et al., 2000). The strongest susceptibility signal maps to the HLA-DRB1 gene in the class II region of the major histocompatibility complex (MHC) (Barcellos et al., 2006; Yeo et al., 2007). Recently, the two largest genome-wide association studies (GWAS) of MS genetics confirmed HLA as the major MS susceptibility locus and provided unequivocal evidence for the association of additional 110 non-MHC "candidate" genetic variants conferring susceptibility to the disease (IMSGC, 2011; IMSGC, 2013).

Notably, the majority of these novel MS-associated genes played pivotal roles in the workings of the immune system and was also associated with other autoimmune diseases, supporting the hypothesis that the same processes occur in different autoimmune diseases (Baranzini et al., 2009; Cotsapas et al., 2011).

To investigate the mechanisms behind the regulation of inflammation in MS, we recently conducted a genome-wide transcriptional analysis of peripheral blood mononuclear cells (PBMC) obtained from treatment-naïve MS women and healthy controls (HC) before, during and after gestation. We identified a MS signature including 347 transcripts differently modulated in MS patients compare to HC before pregnancy (Gilli et al., 2010). Among these, in this work we focused on those genes identified as novel MS risk loci in the GWAS studies (IMSGC, 2011; IMSGC, 2013). This approach highlighted 5 matching genes namely as tumor necrosis factor alpha-induced protein 3 (TNFAIP3), BTB and CNC Homology 1 basic leucine zipper transcription factor 2 (BACH2), prostaglandin E receptor 4 (Subtype EP4) (PTGER4), regulator of G-protein signaling 1 (RGS1), and zinc finger protein 36-C3H-type-like 1 (ZFP36L1). Interestingly, all these transcripts were previously reported as involved in the immune system regulation, mainly with an anti-inflammatory

role (Hollinger et al., 2002; Murn et al., 2008; Yao et al. 2009; Esaki et al., 2010; Sanduja et al., 2011; Roychoudhuri et al., 2013) and associated to several autoimmune diseases (Hunt et al., 2008; Medici et al., 2014; Perdigones et al., 2010; Grant et al., 2009).

Since in our pilot study TNFAIP3 and ZFP36L1 expression reverted to normal in pregnant MS women, their levels were further investigated in a second population. Only TNFAIP3 deregulation was confirmed (Gilli et al., 2010) and subsequently validated in a larger study including also males, showing a correlation between TNFAIP3 levels and the disease clinical course (Gilli et al., 2011).

Here, we aimed to analyzed gene expression of the remaining genes BACH2, PTGER4, and RGS1 in treatment-naïve MS patients compared to HC. Based on recent encouraging genetic association data (IMSGC, 2011; IMSGC, 2013), we decided to include again ZFP36L1 since it was only studied in a small pre-pregnancy female population.

2. Materials and methods

2.1 Enrolled subjects. Clinical and demographic features of MS patients and HC are summarized in Table 1. 49 treatment-naive patients with newly diagnosis of relapsing-remitting MS (RRMS) according to the McDonald criteria (McDonald et al., 2001) and 47 HC were enrolled after giving written consent. Blood samples were obtained during a 2-years period.

This study was approved by Piedmont and San Luigi University Hospital Ethical Committee.

2.2 RNA extraction and real-time PCR analysis. Whole blood samples, collected into a Tempus vacuette, were extracted using the ABI Prism 6100 Nucleic Acid Prep Station (Life Technology Monza, Italy), following the manufacturer's instructions. Total RNA was reverse-transcribed at final concentration of 10 ng/μL using random hexamer primers. Gene expression analysis was performed by real-time PCR using Applied Biosystems' TaqMan gene expression products (Life Technology). Transcriptional expression was normalized using glyceraldehyde-3-phosphate dehydrogenase as reference gene. Expression levels of target genes were calculated by the normalized comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$), using the Universal Human Reference RNA (Stratagene, Santa Clara, California) as calibrator.

2.3 Clinical correlation. Patients were clinically monitored at the MS Center of the San Luigi University Hospital. Gene expression levels were correlated with the time span between the disease onset and the pharmacological therapy initiation, the relapse rate (RR) in the year before the diagnosis and during the follow-up and the Expanded Disability Status Scale (EDSS) score at the time of sampling.

2.4 Statistical analysis. Continuous data are presented as medians and ranges or interquartile ranges. Discrete data are given as counts and percentages. Chi-square tests were performed to compare groups of categorical data; the Mann-Whitney U test was used to compare continuous data.

Regression models were run to evaluate the association between the presence of the disease, adjusted by sex and age, and gene expression levels. To account for non-normality, log or inverse-gaussian or Gamma link functions were chosen according to the Akaike Information Criterion. Associations between expression levels of target genes and clinical parameters were also assessed. Statistical significance was considered at $p<0.05$. All analyses were carried out using R version 3.02.

3. Results

Expression analysis of target genes was performed in whole blood obtained from 49 untreated RRMS patients and 47 HC. There were no statistical differences regarding age and gender between the two groups.

Lower transcript levels of BACH2, PTGER4 and ZFP36L1 were observed ($p=0.017$, $p=0.006$ and $p=0.016$, respectively) in MS patients with respect to HC (Figure 1), according to our previous data (Gilli et al., 2010). Conversely, no statistical significant differences between the two groups were determined for RGS1 (Figure 1), while its expression in both MS and HC population increased with age ($p=0.037$) (data not shown). On the contrary, ZFP36L1 expression significantly decreased with age ($p=0.035$) (data not shown). This result could explain why ZFP36L1 down-regulation was not

validated in our previous study (Gilli et al., 2011) based on not age-adjusted analyses. No sex-related differences in gene expression were highlighted for any gene considered.

A correlation between gene expression and clinical features in MS patients was performed. Patients showed a weak negative correlation between BACH2 expression and the EDSS score ($p=0.045$, $R=0.095$) (data not shown). There were no differences between clinical parameters and the expression of the other analyzed genes, perhaps due to the short follow-up.

4. Discussion

In the present work, we analyzed the expression of novel MS-associated genes and we demonstrated that BACH2, PTGER4 and ZFP36L1 are down-regulated in MS patients' blood cells. Interestingly, all these genes are involved in the immune system regulation with predominant anti-inflammatory role and in the development of autoimmune diseases (Hollinger et al., 2002; Yao et al. 2009; Sanduja et al., 2011; Roychoudhuri et al., 2013; Hunt et al., 2008; Medici et al., 2014; Perdigones et al., 2010; Grant et al., 2009).

BACH2 was demonstrated to be required for efficient formation of T regulatory cells (Treg) (Roychoudhuri et al., 2013), whose immune-modulatory functions are impaired in MS (Huan et al., 2005; Carbone et al., 2014). In addition, BACH2 constrained differentiation of T cell subsets within Th1, Th2 and Th17 lineages. These findings identified BACH2 as a key regulator of CD4+ T-cell differentiation that prevents inflammatory disease by controlling the balance between tolerance and immunity (Roychoudhuri et al., 2013). Consistently, BACH2 variants were linked to several autoimmune diseases including vitiligo, celiac disease, type 1 diabetes (Grant et al., 2009) and recently MS (IMSGC, 2011; IMSGC, 2013).

The second gene investigated, PTGER4, encoding for EP4, one of the four prostaglandin E2 (PGE2) receptors, displays a not well defined role in inflammation. Traditionally, it was considered an immunosuppressant due to its inhibitory function on T cell activation (Murn et al., 2008). However, several groups demonstrated that PGE2 facilitates Th17 expansion and Th1 differentiation, functioning as a mediator of immune inflammation (Yao et al. 2009). Finally,

studies on MS murine model revealed a dual action of PTGER4. In fact, the administration of a EP4 antagonist in the pre-clinical phase suppressed disease progression with concomitant inhibition of Th1 and Th17 cell development, while its administration at the disease onset had little effect. Conversely, EP4 agonist markedly reduced disease severity (Esaki et al., 2010).

The last down-regulated gene, ZFP36L1, is involved in mRNA rapid degradation and translational repression. Through its ability to bind and target AU-rich element (ARE) motifs-containing mRNAs, this protein limits the expression of a number of critical genes, thereby exerting anti-inflammatory and anti-cancer effects (Sanduja et al., 2011).

The regulator of G-protein signaling 1, known as RGS1, is involved in the trafficking of Treg and other immune cells by restricting G-protein signaling duration (Hollinger et al., 2002). Although RGS1 variants were associated with autoimmune diseases as arthritis and psoriasis (Hunt et a., 2008), an alteration of its gene expression was not observed in this work. However, the whole blood analysis could mask a possible altered expression in specific cell subpopulations.

5. Conclusion

The above mentioned genes identified as down-regulated in the present work exert an anti-inflammatory role in the immune system. Taken together, these findings corroborate our initial statement (Gilli et al., 2011) that MS arises from a deregulation of braking signals in inflammation, rather than merely from an overactive pro-inflammatory reaction.

AKNOWLEGMENTS

We would like to thank Rita Guerrieri, Marina Panealbo, Giuliana Savoldi and Angela Zaccaria for their nursing assistance during our study. We also thank Anna Messina and Daniele Dell'Anna for their excellent administrative support.

References

Baranzini SE The genetics of autoimmune diseases: a networked perspective. Curr Opin Immunol. 2009; 21(6):596-605.

Barcellos LF, Sawcer S, Ramsay PP *et al.* Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. Hum Mol Genet 2006; 15:2813-2824.

Carbone F, De Rosa V, Carrieri PB, Montella S, Bruzzese D, Porcellini A *et al.* Regulatory T cell proliferative potential is impaired in human autoimmune disease. Nat Med 2014; 20(1):69-74.

Cotsapas C, Voight BF, Rossin E, *et al.* Pervasive sharing of genetic effects in autoimmune disease. PLoS Genet 2011; 7(8):e1002254.

Ebers GC, Yee IM, Sadovnick AD, Duquette P Conjugal multiple sclerosis: population-based prevalence and recurrence risks in offspring. Canadian Collaborative Study Group. Ann Neurol 2000; 48:927-931.

Esaki Y, Li Y, Sakata D, Yao C, Segi-Nishida E, Matsuoka T *et al.* Dual roles of PGE2-EP4 signaling in mouse experimental autoimmune encephalomyelitis. Proc Natl Acad Sci USA 2010; 107(27):12233-8.

Gilli F, Lindberg RLP, Valentino P, Marnetto F, Malucchi S, Sala A, Capobianco M, di Sapio A, Sperli F, Kappos L, Calogero RA, Bertolotto A Learning from Nature: Pregnancy Changes the Expression of Inflammation-Related Genes in Patients with Multiple Sclerosis. PlosOne 2010; 5(1):e8962.

Gilli F, Navone ND, Perga S, Marnetto F, Caldano M, Capobianco M, Pulizzi A, Malucchi S, Bertolotto A Loss of braking signals during inflammation: a factor affecting the development and disease course of multiple sclerosis. Arch Neurol 2011; 68(7):879-88.

Grant SF, Qu HQ, Bradfield JP, Marchand L, Kim CE, Glessner JT *et al.* Follow-up analysis of genome-wide association data identifies novel loci for type 1 diabetes. Diabetes 2009; 58(1):290-5.

Hollinger S, Hepler JR Cellular regulation of RGS proteins: modulators and integrators of G protein signaling. Pharmacol Rev 2002; 54:527-59.

Huan J, Culbertson N, Spencer L, Bartholomew R, Burrows GG, Chou YK *et al* Decreased FOXP3 Levels in Multiple Sclerosis Patients. Journal of Neuroscience Research 2005;81:45–52 (2005).

Hunt KA, Zhernakova A, Turner G, Heap ARG, Franke L, Bruinenberg M *et al.* Novel celiac disease genetic determinants related to the immune response. Nat Genet 2008; 40(4): 395–402.

International Multiple Sclerosis Genetics Consortium (IMSGC) *et al.* Analysis of immune-related loci identifies 48 new susceptibility variants for multiple sclerosis. Nat Genet 2013; 45(11):1353-60.

International Multiple Sclerosis Genetics Consortium (IMSGC), Wellcome Trust Case Control Consortium , Sawcer S, Hellenthal G. Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 2011; 476(7359):214-9.

McDonald WI, Compston A, Edan G, Polman *et al.* Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis. Ann Neurol 2001; 50(1):121-7.

Medici M, Porcu E, Pistis G, Teumer A, Brown SJ, Jensen RA *et al*. Identification of Novel Genetic Loci Associated with Thyroid Peroxidase Antibodies and Clinical Thyroid Disease. Plos Genet 2014;10(2):e1004123.

Murn J, Alibert O, Wu N, Tendil S, Gidrol X Prostaglandin E2 regulates B cell proliferation through a candidate tumor suppressor, Ptger4. J Exp Med 2008; 22;205(13):3091-103.

Perdigones N, Martín E, Robledo G, Lamas JR, Taxonera C, Díaz-Rubio M et al. Study of chromosomal region 5p13.1 in Crohn's disease, ulcerative colitis, and rheumatoid arthritis. Hum Immunol 2010; 71(8):826-8.

Robertson NP, Clayton D, Fraser M, Deans J, Compston DA Clinical concordance in sibling pairs with multiple sclerosis. Neurology 1996 ; 47(2):347-52.

Roychoudhuri R, Hirahara K, Mousavi K, Clever D, Klebanoff CA *et al.* BACH2 represses effector programs to stabilize T(reg)-mediated immune homeostasis. Nature 2013; 27;498(7455):506-10.

Sanduja S, Blanco FF, Dixon DA The roles of TTP and BRF proteins in regulated mRNA decay. Wiley Interdiscipl Rev RNA 2011; 2(1):42-57.

Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K *et al.* Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med. 2009; 15(6):633-40.

Yeo TW, De Jager PL, Gregory SG A second major histocompatibility complex susceptibility locus for multiple sclerosis. Ann Neurol 2007; 61(3):228-36.

Figure
Click here to download high resolution image

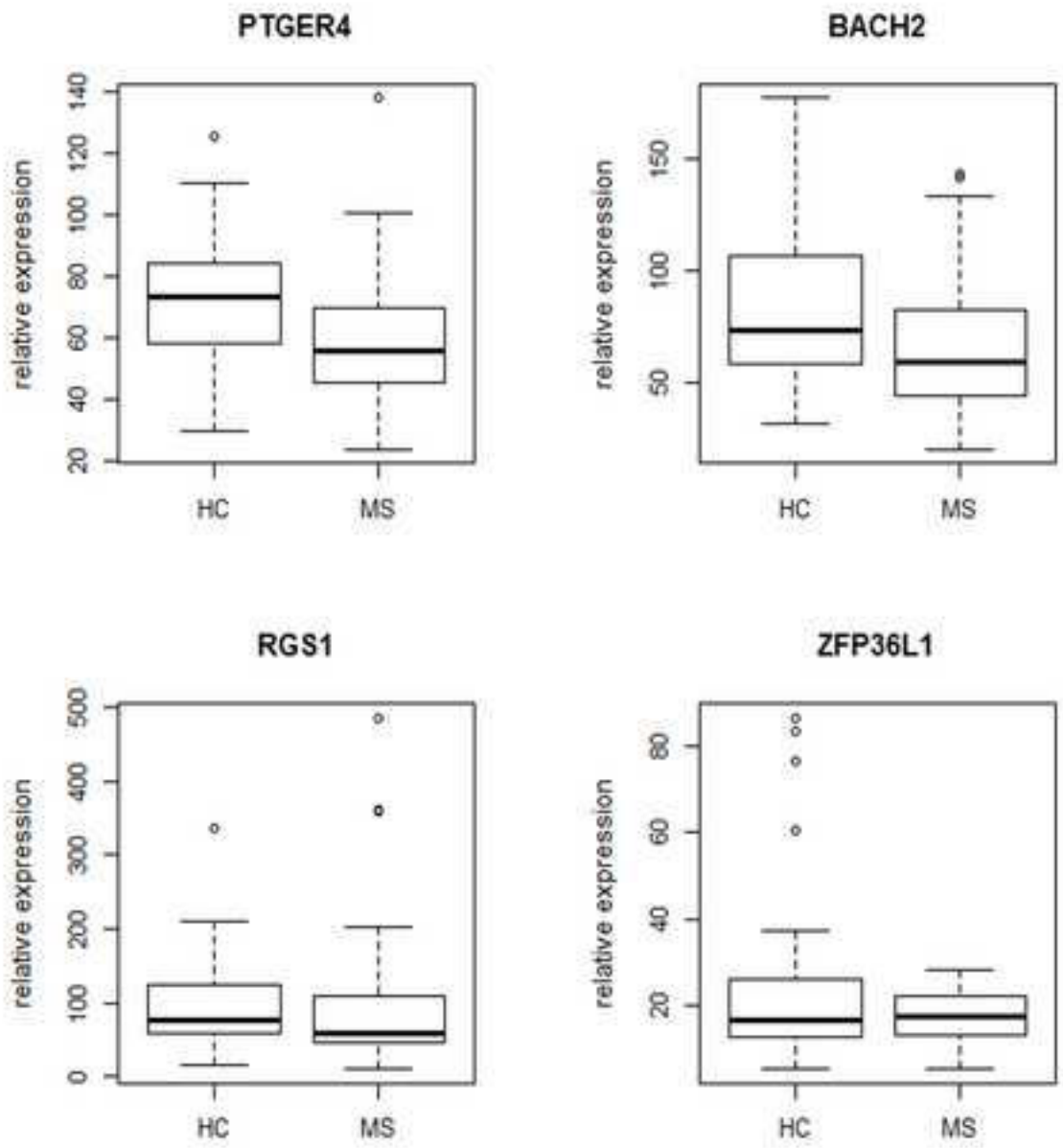

Figure legends

Figure 1. Whole blood gene expression levels in MS patients and HC. Comparison of median gene expression levels of (A) PTGER4, (B) BACH2, (C) RGS1, (D) ZFP36L1 between 47 HC and 49 treatment-naive MS patients. The regression analysis adjusted for sex and age disclosed that PTGER4, BACH2 and ZFP36L1 were down-regulated in MS patients compared to HC (p= 0.006, p= 0.017 and p= 0.016, respectively). No differences were detected for RGS1. Relative expression was calculated by the normalized comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$).

Tables

Table 1. **Clinical and demographical characteristics of MS patients and HC.**

Characteristics	**HC**	**MS patients**	**p value**
Sample size, n	47	49	
Women, % (n)	53 (25)	63 (31)	0,32 [a]
Age, median (interquartile range)	32 (27, 46)	39 (28, 46)	0,28 [b]
Disease duration at start of therapy, months, median (interquartile range)		17 (10, 72)	
Follow up, months, median (interquartile range)		19 (16, 21)	
RR one year before therapy, median (range)		1 (0, 3)	
RR in the follow up, median (range)		0 (0, 6)	
EDSS at start of therapy, median (range)		1 (0, 6.5)	

[a] chi-square test, [b] Mann–Whitney U test. Abbreviations: RR= relapse rate; EDSS= Expanded Disability Status Scale.