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Methylation-dependent *PAD2* upregulation in multiple sclerosis peripheral blood

Roberta Calabrese[1,2*], Michele Zampieri[1,2*], Rosella Mechelli[3], Viviana Annibali[3], Tiziana Guastafierro[1,2], Fabio Ciccarone[1,2], Giulia Coarelli[3], Renato Umeton[3], Marco Salvetti[3] and Paola Caiafa[1,2]

Abstract

Background: Peptidylarginine deiminase 2 (PAD2) and peptidylarginine deiminase 4 (PAD4) are two members of PAD family which are over-expressed in the multiple sclerosis (MS) brain. Through its enzymatic activity PAD2 converts myelin basic protein (MBP) arginines into citrullines – an event that may favour autoimmunity – while peptidylarginine deiminase 4 (PAD4) is involved in chromatin remodelling.

Objectives: Our aim was to verify whether an altered epigenetic control of PAD2, as already shown in the MS brain, can be observed in peripheral blood mononuclear cells (PBMCs) of patients with MS since some of these cells also synthesize MBP.

Methods: The expression of most suitable reference genes and of *PAD2* and *PAD4* was assessed by qPCR. Analysis of DNA methylation was performed by bisulfite method.

Results: The comparison of *PAD2* expression level in PBMCs from patients with MS vs. healthy donors showed that, as well as in the white matter of MS patients, the enzyme is significantly upregulated in affected subjects. Methylation pattern analysis of a CpG island located in the *PAD2* promoter showed that over-expression is associated with promoter demethylation.

Conclusion: Defective regulation of PAD2 in the periphery, without the immunological shelter of the blood–brain barrier, may contribute to the development of the autoimmune responses in MS.

Keywords

DNA Methylation, epigenetics, multiple sclerosis, *PAD2*

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Introduction

Multiple sclerosis (MS) is a major, often disabling, neurological disease affecting young adults.[1] The damage to the central nervous system (CNS) results from inflammation and neurodegeneration, but the definition of what triggers and sustains both mechanisms, within or outside the CNS, remains incomplete. Structural and quantitative changes in myelin proteins may support the pathogenesis of the disease through various mechanisms, including an autoaggressive immune response to myelin.[2,3] Individuals with MS show a diffuse hypomethylation of the genome in the white matter.[3] This reprogramming of the DNA methylation pattern may be dangerous as, apart from inducing genome destabilization, it can reactivate genes that should remain silent in the brain.[4-6] In this scenario, an example of gene reactivation dependent on DNA methylation pattern is peptidylarginine deiminase 2 (*PAD2*), whose promoter has been found to be less

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Table 1. Demographic and clinical data of patients with multiple sclerosis

Number	32
Female/Male	22/10
Age (years)	36.6 (9.6)
Disease-type	31 RR; 1 SP
Disease duration (range-years)	0.1–18
EDSS median (range)	1.5 (1–2)
MRI	28 inactive; 4 active
Disease-modifying therapies	None

RR, Relapsing–remitting; SP, Secondary progressive; MRI, magnetic resonance imaging; EDSS, Expanded Disability Status Scale

methylated in the white matter of subjects with MS.[3] This was suggested as the mechanism leading to the over-expression of the enzyme, with a consequent increase of citrullines on myelin basic protein (MBP), the best substrate of *PAD2* and a putative autoantigen in MS. Citrullinated isoforms of MBP may reduce the stability of myelin[3,6,7] and may threaten the maintenance of the immunological tolerance to MBP. However, it is unclear how changes of this kind, occurring behind the blood–brain barrier, may lead to the immunization against myelin autoantigens. One possibility may be a priming against a citrullinated MBP in the periphery. Since MBP is indeed also expressed in lymphoid tissues,[8] we verified whether an altered epigenetic control of *PAD2*, as already shown in the MS brain, can be observed in peripheral blood mononuclear cells (PBMCs) of patients with MS. We extended the analyses to the peptidylarginine deiminase 4 (*PAD4)* gene, another member of the PAD family, which codes for an enzyme involved in chromatin remodelling[9,10] and is highly expressed in the brain of patients with MS.[11] Moreover, like *PAD2*, *PAD4* is expressed in peripheral blood cells and is able to deiminate MBP arginine residues.[11-13]

Subjects and methods

Patients and healthy donors

We analysed freshly isolated PBMCs from 32 patients with definite MS and from 30 healthy volunteers (50% women). All affected individuals were treatment naïve. All samples were drawn at least 3 months after the last steroid therapy. A contrast-enhanced magnetic resonance imaging (MRI) scan was obtained in MS patients within 24 h from sampling. Demographic and clinical details of MS patients are summarized in Table 1.

Procedures

PBMCs were obtained by gradient centrifugation through 'Lymphoprep' solution (Axis-Shield). RNA (from 3×10^6 cells) was isolated by TRIZOL reagent (Invitrogen) adopting the manufacturer's protocol, and quantified by NanoDrop (NanoDrop Technologies). RNA quality was evaluated by capillary chip electrophoresis using the Agilent 2001 Bioanalyzer (Agilent Technologies). Reverse transcription and quantitative Taqman RT-PCR were carried out as reported previously.[14] The following TaqMan Gene Expression Assays IDs for *PAD2* and *PAD4* were used: Hs00247108_m1 and Hs00202612_m1. DNA methylation analysis of the *PAD2* promoter was performed by bisulfite sequencing using previously reported primers.[15]

Statistical analysis

Expression data were analysed by two different statistical algorithms: geNorm and NormFinder.[16,17] GeNorm uses an algorithm to calculate the M value, a gene expression stability measure, defined as the mean pairwise variation for a given gene compared with the remaining tested genes. Hence, a lower value of M indicates higher stability of the reference gene. We considered 1.5 as a cut-off for the M value; genes with an M value above this value are considered unreliable for normalization. The programme also establishes a rank order of gene stability via stepwise exclusion of the least stable gene, which allows identification of the remaining two genes with the lowest M value. To determine how many reference genes should be used for accurate normalization, geNorm also performs a stepwise calculation between sequential normalization factors (NF). It starts to calculate the pairwise variation V2/3 between the NF2 (including the two most stable reference genes) and the normalization factor NF3 (including the three most stable reference genes). It then performs a stepwise calculation of the Vn/n+1 between the NFn and the NFn+1. A variation of the Vn/n+1 above 0.15 indicates that the inclusion of an additional reference gene is required.

NormFinder provides a ranking of the tested genes based on a direct measure of both the overall variation of expression of candidate reference genes in the samples group and the variation between samples subgroups. The combined measure of intra- and inter-group variation is given as a stability value, which is an estimation of the variation in expression of candidate reference genes. Low stability values define genes showing high stability of expression. In our settings the intra-group variation is calculated across all samples, while the inter-group is calculated between the control and MS groups of samples.

We adopted Pearson product-moment correlation coefficient (r) in order to obtain a quantitative estimation about a possible linear dependency among gene expression and associated clinical data.

Results

Choice of the most suitable reference genes

In order to perform gene expression analysis on PBMC samples derived from patients with MS we first searched for a reference gene suitable for normalization. We selected gene

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Table 2. Panel of candidate reference genes evaluated in this study

Symbol	Gene name	TaqMan assays ID
GUSB	Glucuronidase β	Hs99999908_m1
ACT	Actin β	4333762
HPRT1	Hypoxanthine guanine phosphoribosyl transeferase 1	Hs99999909_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Hs99999905_m1
18S	18s rRna	Hs99999901_m1

transcripts with different physiological roles (Table 2) to minimize the risk of the MS affecting all of the genes tested. The non-normalized expression levels showed normal distribution (Shapiro–Wilk test). *18s* rRNA and *ACTB* showed a significant downregulation in MS samples, while the expression of the other candidate reference genes remained comparable between normal and MS samples (Figure 1A).

We applied two different statistical algorithms, NormFinder and geNorm, to the same data set to evaluate the most stable reference genes. As shown in Figure 1B, *GUSB* presented the highest stability in the ranking of candidate housekeeping genes according to NormFinder analysis.

As the NormFinder analysis could be affected by sampling errors which enhance experimental component of variation, to gain an unbiased estimation of the stability of the selected candidate reference genes, geNorm algorithm was also adopted. It is based on the assumption that the ratio between two putative reference genes is nearly constant across samples independently of RNA amount analysed per sample. The ranking of the tested genes according to geNorm expression stability value (M) indicated *GUSB* and *HPRT1* as the most stable pair of genes (Figure 1C). The use of more than the two most stable reference genes identified (*GUSB* and *HPRT1*) was not required (Figure 1D).

Gene expression profiling in PBMCs

Over-expression of the *PAD2* gene is believed to be one of the factors involved in MS disease. We assessed the expression level of this gene in PBMCs of healthy and MS subjects by qRT-PCR to verify whether the upregulation of the *PAD2* gene, observed in the brain of patients affected by MS, could

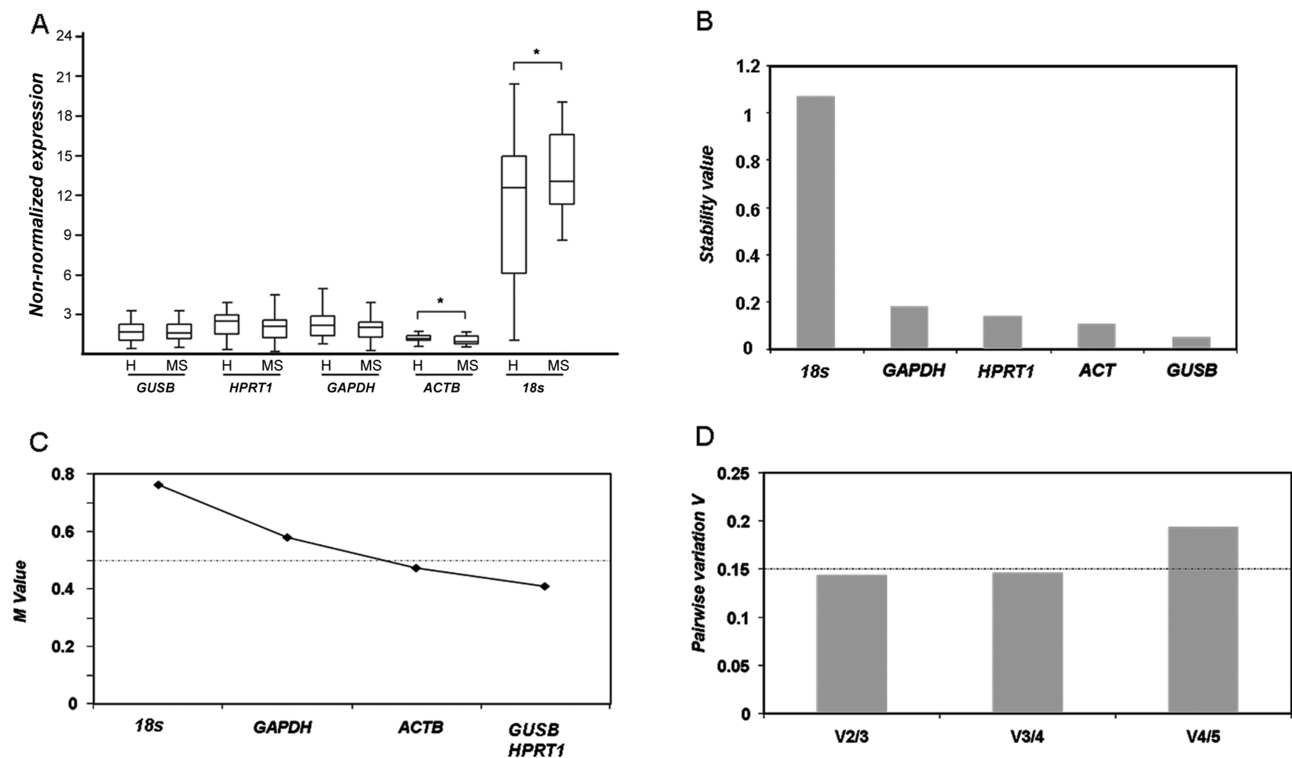

Figure 1. Expression stability of candidate reference genes
(A) Expression levels of candidate genes starting from equal amounts of RNA obtained from peripheral blood mononuclear cells (PBMCs) of healthy and multiple sclerosis (MS) subjects are shown as medians (horizontal line), 25th to 75th percentile (boxes) and range (whiskers). *$p \leq 0.05$
(B) Expression stability value calculated by NormFinder for each candidate reference gene comparing normal vs. MS samples. A lower value indicates higher expression stability.
(C) Selection of the best pair of candidate reference genes for accurate normalization by geNorm. The average expression stability M of candidate reference genes during stepwise exclusion of the least stable gene is shown. The *X*-axis indicates genes ranked according to their expression stability measure. The *Y*-axis indicates the average M-value of the remaining genes after having excluded the indicated genes (i.e. the less stable).
(D) Pairwise variation between two sequential normalization factors (NF_n and NF_{n+1}) to determine the optimal number of reference genes for reliable normalization.

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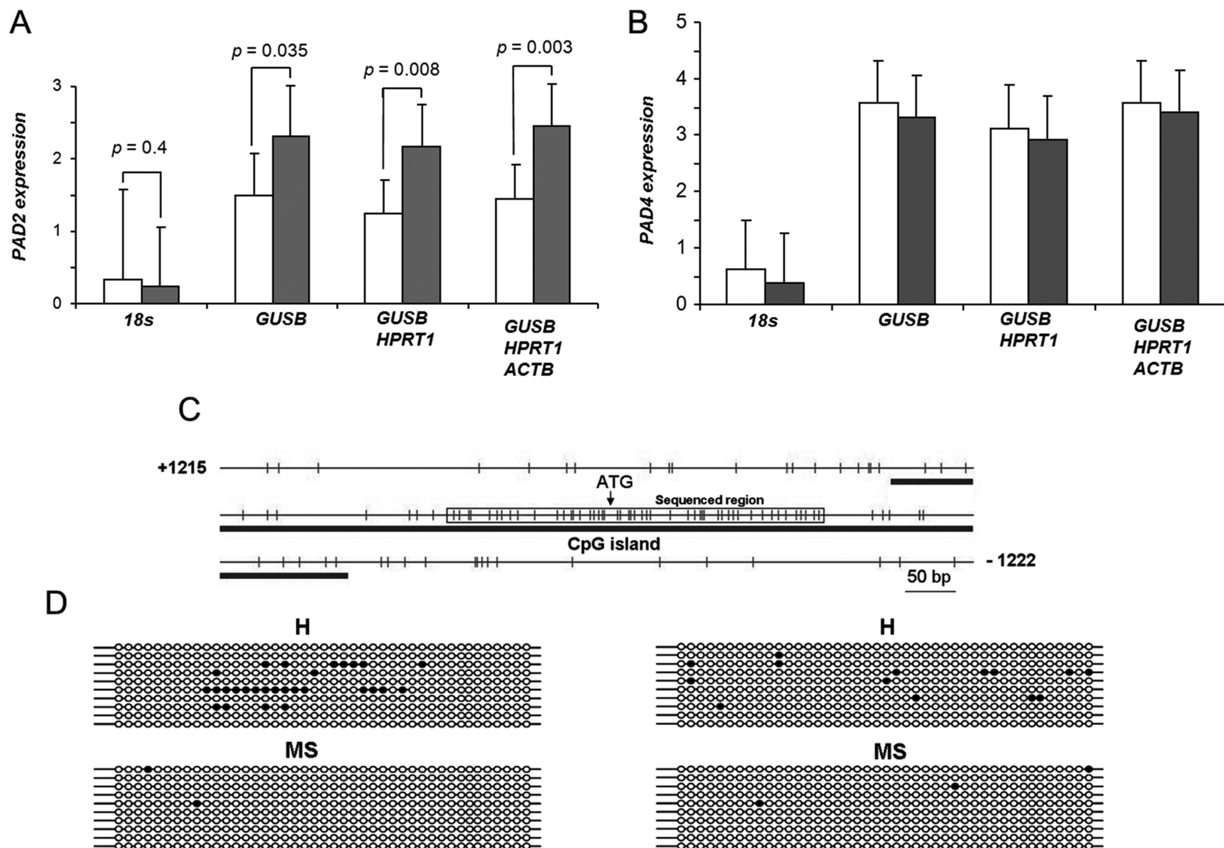

Figure 2. *PAD2* and *PAD4* expression in normal and multiple sclerosis peripheral blood mononuclear cell samples and methylation profiling of *PAD2* promoter

PAD2 (A) and *PAD4* (B) expression measured by qRT-PCR normalized by single or multiple reference genes as indicated by geNorm and NormFinder (mean ±C.V.).

(C) Graphical representation of the CpG island (grey line) in the promoter region of *PAD2*. Numbers indicate the distance in base pairs from the first codon; dashes: CpG dinucleotides.

(D) The methylation state of *PAD2* promoter region was evaluated by bisulfite sequencing. Up to 10 independent clones for two multiple sclerosis (MS) and two control samples (H) were analysed by the sequencing procedure. Each row of circles represents the sequence of an individual clone. Open circle, unmethylated CpG site; filled circle, methylated site.

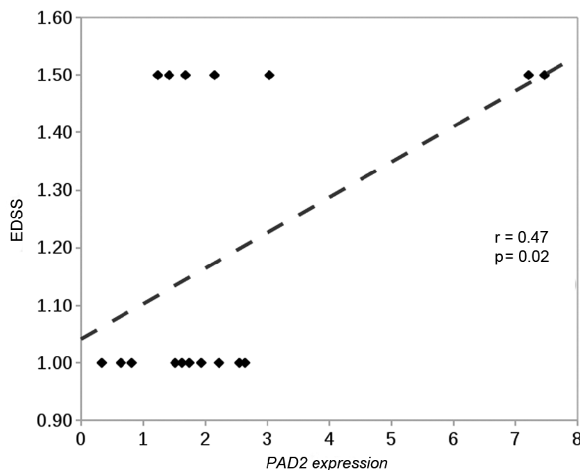

Figure 3. Pearson product-moment linear coefficient analysis of the relation between *PAD2* expression and Expanded Disability Status Scale (EDSS). r = correlation coefficient, $p < 0.05$

be also evident in blood cells. Analysis was extended also to *PAD4*, another member of PAD family whose level is increased in the brain white matter of patients with MS.[11]

As shown in Figure 2A, we evidenced a significant increase in *PAD2* mRNA levels in patients with MS compared with healthy donors only after normalization with the most stable housekeeping genes or with their geometric mean as predicted by both NormFinder and geNorm analysis. This is the first evidence reporting that *PAD2* mRNA is also upregulated in mononucleated blood cells. The analysis of *PAD4* expression did not reveal any significant change (Figure 2B).

Methylation profiling of PAD2 promoter in PBMCs

Demethylation of CpGs located in the promoter of *PAD2* gene (Figure 2C) is considered the principal event in

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inducing the upregulation in the brains of patients with MS.[3] We verified by bisulfite sequencing whether demethylation also occurred on the *PAD*2 promoter in the PBMCs of MS patients. The analyses, performed on a DNA fragment of about 400 bp containing 48 CpGs, were carried out on two randomly chosen MS patients compared with healthy donors matched for gender and age. As shown in Figure 2D, the *PAD*2 promoter region of MS patients was extensively demethylated, supporting the observed *PAD*2 gene upregulation. We note that the analysis of the methylation patterns of healthy donors showed that methylation was present only in a part of the examined clones.

Correlation between PAD2 gene expression profiling and clinical data

We evaluated the Pearson product-moment coefficient in order to assess a possible correlation between *PAD2* expression level and clinical data available, in terms of disease duration (DD), Expanded Disability Status Scale (EDSS), and MRI activity (as reported in Table 1). We observed no linear correlations analysing the entire patient population available, and additionally, no correlations were found partitioning the sample according to the gender.

We further looked into the data in order to investigate whether a subset of patients might exist where the linear correlation hypothesis might hold. We identified a subset of patients ($n = 19$) with inactive MRI, EDSS value < 2, and not containing the two worst data outliers. With respect to this subset, which is 63% of the patients, we observed a medium correlation between *PAD2* levels and EDSS (the correlation coefficient is 0.47 and its associated p-value is 0.02), as reported in Figure 3.

Discussion

PAD2 and PAD4, two members of PAD family[18] drew our attention as they were found present in a higher quantity in the CNS white matter of patients with MS vs. healthy individuals.[11] The high level of *PAD2* expression is not present in other neuropathologies,[3] suggesting an alteration that may be specific for MS. In accord with this possibility, MBP is the best PAD2 substrate. PAD2 and PAD4, converting arginine into citrulline, insert a post-synthetic modification onto their protein substrates.[18] The decreased positive charge on MBP, that follows the citrullination reaction, destabilizes the interaction between the lipid bilayer and myelin, and affects myelin structure.[3,6,7] This excessive deimination of MBP changes the protein's three-dimensional structure, increasing its susceptibility to some proteases.[19] This process may enhance the production of citrullinated epitopes, with an intrinsic risk of a breakdown of the immunological tolerance to MBP. Indeed, citrullinated MBP peptides can induce and sustain inflammation in experimental autoimmune encephalomyelitis (EAE).[20] However, it is unclear how the production of such modified epitopes, occurring behind the blood–brain barrier, can lead to a sensitization of the immune system in the periphery. Previous work has demonstrated that there are opportunities for this to happen: during EAE the expression of MBP is increased in lymphoid tissue,[21] and patients with MS may have an enhanced T-cell reactivity to citrulline-containing MBP.[22]

Despite the limited sample size, here we show that in PBMC from patients with MS, the hypomethylation of the *PAD2* promoter seems to account for the increased 'peripheral' *PAD2* expression. This event may set the stage for an increased reactivity or 'epitope spreading' towards citrullinated MBP sequences. In this context it is interesting to note that the increased *PAD2* expression in the PBMCs of patients with MS may not be mirrored by a similar upregulation in the thymus,[15] hence favouring an imbalance between the probabilities of tolerization vs. those of immunization. Finally, citrullinated MBP epitopes may be more potent than non-citrullinated ones in inducing a pro-inflammatory TH1 polarization in patients with MS.[23] On the other hand, the humoral IgG response to citrullinated MBP does not seem to discriminate patients from controls.[24] Unless a subgroup of MS patients with positive IgG responses to citrullinated epitopes is identified in larger studies, the reactivity to such targets, in the B-cell compartment, does not repeat the findings in rheumatoid arthritis (RA) where antibodies to citrullinated peptide antigens discriminate two major subsets of patients with some differences also in aetiological features.[25]

Concerning PAD4, recent findings show that this isoform is also upregulated in myelin in MS brain, and is able to catalyze MBP deimination *in vitro*,[11] suggesting a possible contribution of PAD4, together with PAD2, in the increase of citrullinated MBP in MS. These data and the fact that *PAD4* is highly expressed in lymphoid tissues[12,13] together with MBP,[8] provided the rationale for determining its expression in PBMC from MS patients. Our results, showing the selective increase in *PAD2* expression, confer more specificity to our findings, which thus point to a distinctive involvement of PAD2.

The role of PAD4 could be different, as its main substrates are H3 and H4 histone proteins[9,10] in the nuclei, and recent data suggest that PAD4 activity is involved in the repression of gene transcription by stabilizing the histone H2A/H2B dimer.[26]

Our data add a new element to the hypothesis of a role for the immune response to citrullinated myelin epitopes in the pathogenesis of MS. Further work on this topic is highly warranted, not only because of the analogies with RA, where this kind of reactivity represents a major pathogenetic (and diagnostic) component, but also considering the increasing awareness about the potential importance of epigenetic mechanisms in the pathogenesis of multifactorial diseases.[27]

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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