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mRNA profilin identifies low levels of phosphatases dual-specific phosphatase-7 (DUSP7) and cell division cycle-25B (CDC25B) in patients with early arthritis

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Summary

Phosphotyrosine phosphatases (PTPs) control phosphorylation levels and, consequently, regulate the output of intracellular signalling networks in health and disease. Despite the high number of PTPs expressed in CD4 T cells and their involvement in autoimmunity, information about the expression profile of PTPs in these cells has not been obtained in patients diagnosed with autoimmune diseases. Here, we compare the expression profile of PTPs in CD4 T cells of healthy volunteers and patients submitted to an early arthritis clinic, due to suspicion of rheumatoid arthritis, an autoimmune disease mediated by CD4 T cells. We found lower transcript levels of the mitogen-activated protein kinase (MAPK) phosphatase dual-specific phosphatase-7 (DUSP7) and the cell division cycle-25B (CDC25B) in T cells of patients. While the low expression level of DUSP7 was restricted to patients with positive rheumatoid factor and anti-citrullinated protein antibodies, the altered expression of CDC25B correlated with the activity of the disease. Low levels of CDC25B might contribute to the progression of the autoimmune arthritis and/or might be consequence of the inflammatory environment in the active disease. The possible role of DUSP7 and CDC25B as biomarkers of the disease in clinical protocols is discussed.

Keywords: CD4 T cells, CDC25B, DUSP7, early arthritis, PTPs

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease that affects approximately 0·5% of the population worldwide and processes with inflammation of synovial joints and bone destruction [1]. RA is a multi-factorial disease resulting from the combination of environmental and genetic factors. Although the aetiology of this disorder seems to be heterogeneous and to involve several cell subsets of the immune system, currently there is compelling evidence indicating that the pathogenesis involves the specific autoimmune response of T cells [2,3]. Underscoring the role of T helper (Th) cells in RA, several alleles of the human leucocyte antigen D-related (HLA-DR) are associated with the disease [4].

Phospho-tyrosine phosphatases (PTPs) constitute a complex family of more than 100 related enzymes classified into four classes: class I, containing the classical PTPs and the dual-specific phosphatases (DSPs); class II, containing the low molecular weight PTP; class III, containing cell division cycle-25 PTPs (CDC25s); and class IV, containing the eyes absent PTPs [5]. Lymphocytes express 60–70 PTPs expected to be critical to balance phosphorylation levels [6], which are essential for a normal immune response while preventing diseases [7,8]. Some alterations in genes coding for classical PTPs that regulate intracellular signalling in T cells have been involved in autoimmunity, including single nucleotide polymorphisms of PTPN22 and PTPN2, aberrant mRNA processing of protein tyrosine phosphatase, receptor type C (PTPRC) and altered expression levels of PTPN6 and PTPN2 [8–11]. None the less, there is no detailed information concerning the expression profiles of PTPs in immune cells involved in human autoimmune diseases.

Due to the relevant role of CD4 T cell immune responses in RA, we aimed to compare the expression profile of PTPs in total peripheral blood CD4 T cells of healthy individuals and patients submitted to an early arthritis (EA) clinic. In order to avoid the potential effects of specific RA therapy in gene expression, we recruited patients before the

Table 1. Demographic and clinical data of the population included in this study

	Healthy donors (n = 16)	RA (n = 30)	UA (n = 12)	Test result
Female, n (%)	11 (79·2)	22 (73·3)	7 (58·3)	n.s.
Age [years; p50 (IQR)]	47 (40·25–61·00)	57·5 (46·75–66·75)	52·5 (40·5–59·75)	n.s.
Smoking, n (%)				n.s.
Never	7 (43·75)	14 (46·67)	7 (58·33)	
Ever	8 (50·00)	11 (36·67)	5 (41·66)	
Current	1 (6·25)	5 (16·67)	–	
Disease duration [months; p50 (IQR)]	–	5·1 (2·8–10·1)	7·2 (2·1–12·2)	n.s.
RF, n (%)	–	18 (60·00)	4 (33·33)	0·06
ACPA, n (%)	–	16 (53·33)	5 (41·66)	n.s.
ANA, n (%)	–	12 (40·00)	5 (41·66)	n.s.
DAS28 [p50 (IQR)]	–	4·1 (3·1–5·7)	3·5 (2·7–4·8)	n.s.
SDAI [p50 (IQR)]	–	19·1 (11·25–35·33)	13·1 (7·8–25·0)	n.s.
HUPI [p50 (IQR)]	–	7 (4–10·3)	5·25 (3·8–10·4)	n.s.
HAQ [p50 (IQR)]	–	0·875 (0·375–1·438)	0·8125 (0·4063–1·531)	n.s.

The *P*-value is shown of Fisher's exact or Mann–Whitney test for comparing qualitative or quantitative variables, respectively. Three groups were compared by the Kruskal–Wallis test. IQR = interquartile range; n.s. = non-significant; RF = rheumatoid factor; ACPA = anti-citrullinated peptide antibodies; ANA = anti-nuclear antibodies; DAS28 = 28-joint Disease Activity Score; SDAI = Simplified Disease Activity Index; HUPI = 'Hospital Universitario de La Princesa' Index; HAQ = Health Assessment Questionnaire.

administration of any drug at their initial visit to the hospital. Insights into the potential role of transcript levels of PTPs as biomarkers of the disease were obtained by comparing seropositive and seronegative patients, as well as patients with different disease activity.

Materials and methods

Patients and clinical parameters

We used 42 patients from the Princesa Early Arthritis Register Longitudinal (PEARL) study, a prospective register of patients submitted to an EA clinic. The protocol includes the collection of sociodemographic, clinical, analytical and therapeutic information, as well as biological samples at five structured visits. Rheumatoid factor (RF) was assessed by nephelometry and anti-citrullinated peptide antibodies (ACPA) and anti-nuclear antibodies (ANA) by enzyme-linked immunosorbent assay (ELISA) with QUANTA Lite CCP3 and ANA kits, respectively (Inova Diagnostics, San Diego, CA, USA). CD4 T cells were isolated during the baseline visit, so only one patient had received treatment (methotrexate) before sample collection.

Of these 42 patients, 30 fulfilled the 2010 American College of Rheumatology/European League Against Rheumatism criteria for RA [12] and 12 were classified as undifferentiated arthritis (UA) [13]. Considering the discrepancies found when assessing the activity of the RA, we categorized global disease activity by combining 28-joint Disease Activity Score (DAS28) [14], 'Hospital Universitario de La Princesa' Index (HUPI) [15] and Simplified Disease Activity Index (SDAI) [16] in the following subsets: remission, low, moderate and high disease activity when the three indices were in agreement, and remission/low, low/moderate and moderate/high disease activity when disagreement was observed. Healthy volunteers (n = 16) were also recruited and all information concerning the population included in the study is shown in Table 1.

This study was conducted according to the recommendations of the Declaration of Helsinki and the protocol approved by the Clinical Research Ethics Committee of the 'Hospital Universitario La Princesa' (Madrid, Spain). All patients signed a written consent before being included into the PEARL study.

Isolation of peripheral blood CD4 T cells

Peripheral blood mononuclear cells were obtained by density gradient centrifugation on Lymphoprep™ (Rafer, Spain). CD4 T cells were isolated using the Dynabeads® Untouched™ human CD4 T cells kit (Invitrogen, Carlsbad, CA, USA). Purities above 95% were typically obtained.

Real-time quantitative polymerase chain reaction (RT–qPCR)

RNA was extracted using the Absolutely RNA Microprep Kit (Agilent Technologies, Santa Clara, CA, USA) and 2 μg were reverse-transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). Using TaqMan low-density arrays (TLDAs, ThermoFisher, Fremont, CA, USA), three healthy donors and three RA patients were compared initially for the expression profiles of genes coding for DSPs and class II, III and IV PTPs, as well as for suppressors of cytokine signalling (SOCS). Age and gender were paired in each comparison performed per TLDA (Fig. 1a). The three patients were diagnosed with seropositive ($ACPA^+/RF^+$) RA and

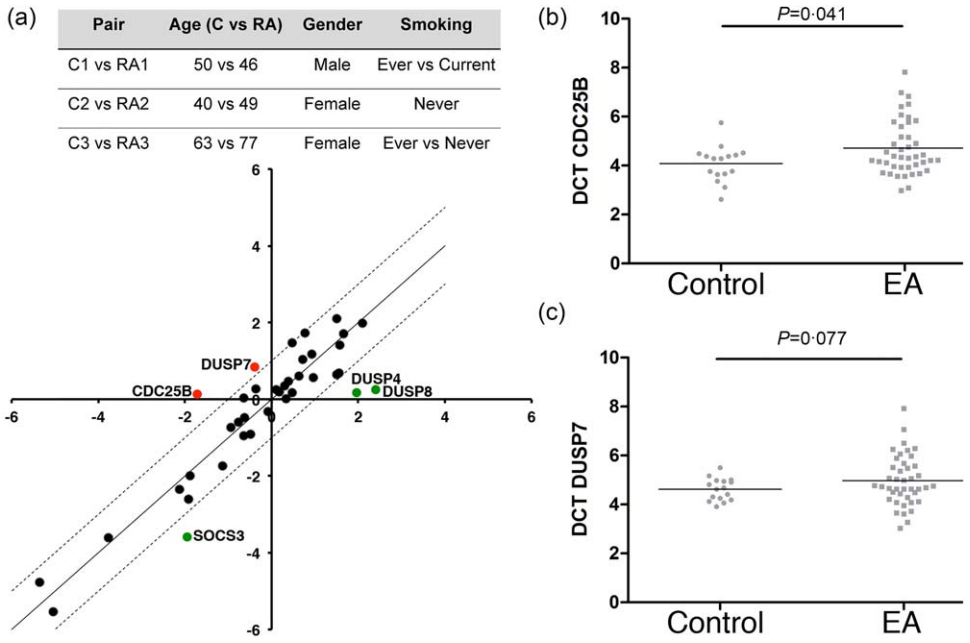

Fig. 1. Analysis of the expression of phospho-tyrosine phosphatases (PTPs) in healthy donors and patients with early arthritis (EA). (a) The table provides information for healthy and diseased donors used in the initial comparisons. Cn and RAn indicate the number of healthy donors and RA patients, respectively. Plot of average dCT values obtained for each gene in the three comparisons of healthy donors (*x*-axis) and rheumatoid arthritis (RA) patients (*y*-axis). Diagonal line labels the position of genes with the same expression. Dashed lines identify genes whose average dCT differs in an absolute value higher than one CT. Genes with lower and higher transcript levels in RA patients are labelled in red and green, respectively. (b,c) Transcript levels of CDC25B (b) and dual-specific phosphatase-7 (DUSP7) (c) in control volunteers and EA patients. Dots in graphs represent the dCT value in each individual analysed. The horizontal line indicates the average value. The probability of the Student's *t*-test is indicated.

had not received any treatment at the time of sample extraction. Genes with CT values > 33 were rejected, and the delta (D)CT was calculated using as reference the average CT of all genes analysed. A difference equal to or higher than 1 CT was indicative of genes with different expression levels.

Data obtained with TLDAs were studied further in higher samples of healthy volunteers and patients submitted to the EA clinic. qPCR reactions were performed with TaqMan Gene Expression Master Mix and the following predesigned qPCR assays (Applied Biosystems): GNB2L1 (Hs00272002_m1), dual-specific phosphatase (DUSP)8 (Hs00792712_g1), DUSP7 (Hs00997002_m1), DUSP4 (Hs01027785_m1) and CDC25B (Hs00244740_m1). GNB2L1 was used as housekeeping gene in these sets of qPCRs.

Statistical analysis

A Student's *t*-test for comparing groups of qPCR data was implemented in GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The Welch correction was applied when variances were different according to a *F*-test. For comparing sociodemographic, clinical and analytical variables, Fisher's exact, Mann–Whitney and Kruskal–Wallis tests were used (Table 1). *P*-values ≤0·05 were considered statistically significant.

Results

Expression profile of PTPs in CD4 T cells of healthy donors and RA patients

Despite the similar expression profile of PTPs found in CD4 T cells from RA patients and healthy donors in the initial comparison (see Materials and methods), the expression level of four PTPs was found to be substantially different (Fig. 1a). The transcript levels of the mitogen activated protein kinase (MAPK) phosphatase (MKP) DUSP7 and the cell division cycle-25B (CDC25B) were substantially lower and the MKPs DUSP8 and DUSP4 substantially higher in RA patients. Expression of the suppressor of cytokine signalling-3 (SOCS-3) was higher in RA patients, consistent with a proinflammatory cytokine environment in the pathology. This result is in agreement with the overexpression of SOCS-3 in T cells of RA patients documented previously [17].

The expression level of these genes was analysed further in a higher sample of healthy donors and patients with EA. While transcript levels of DUSP4 and DUSP8 were not different (data not shown), a significantly lower expression of CDC25B was found in CD4 T cells obtained from patients (Fig. 1b). A lower expression of DUSP7 was also observed,

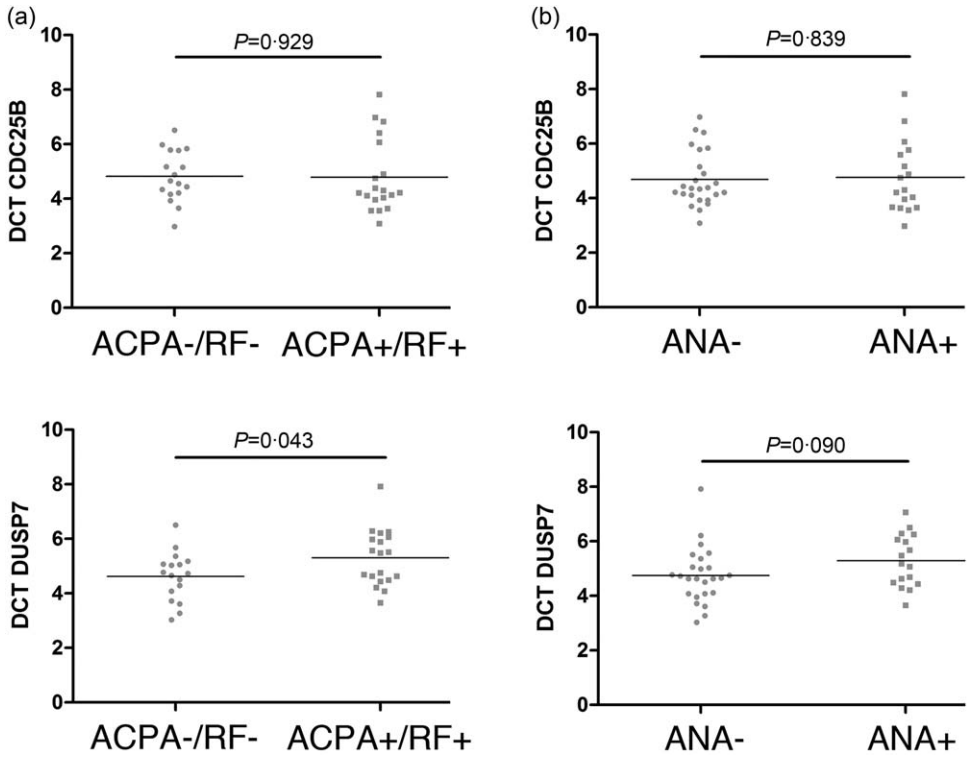

Fig. 2. Low expression of dual-specific phosphatase-7 (DUSP7) in anti-citrullinated peptide antibodies $(ACPA)^+$/rheumatoid factor $(RF)^+$ seropositive patients. (a,b) Graphs represent the dCT value of CDC25B or DUSP7 in EA patients seronegative or seropositive for ACPA and RF or anti-nuclear antibodies (ANA). The horizontal line indicates the average value. The probability of the Student's *t*-test is indicated.

although the difference was not statistically significant (Fig. 1c). Transcript levels of CDC25B and DUSP7 were not different in cells of patients classified as RA and UA (Supporting information, Fig. S1). We found neither correlation between the expression level of CDC25B or DUSP7 and the age of donors nor the genes showing expression levels associated with gender or smoking habit (data not shown).

Lower transcript levels of DUSP7 in seropositive ($ACPA^+/FR^+$) patients

We also studied the relationship of the expression levels of CDC25B or DUSP7 with the serum title of RF, ACPA and ANA. The expression level of DUSP7, but not of CDC25B, was significantly lower in seropositive ($ACPA^+/FR^+$) than in seronegative patients (Fig. 2a). Lower transcript levels of DUSP7 were also found in ANA^+ patients, although differences were not statistically significant (Fig. 2b). In any case, correlation was found with the title of the sera (data not shown). No significant difference in CDC25B was found depending on the ANA status (Fig. 2b).

Low expression levels of CDC25B correlated with the disease activity

We studied the correlation of DUSP7 or CDC25B transcript levels with the global disease activity determined by combining the DAS28, HUPI and SDAI clinical parameters (see Material and methods). The expression of DUSP7 and CDC25B was analysed in the different subsets of disease activity, including remission (R), remission/low (R/L), low (L), low/moderate (L/M), moderate (M), moderate/high (M/H) and high (H). The expression level of CDC25B in patients with active disease was lower than in patients in remission groups or in healthy volunteers ($<dCT>_{active} = 4{\cdot}90 \pm 1{\cdot}17$, $<dCT>_{remission} = 3{\cdot}93 \pm 0{\cdot}25$ and $<dCT>_{controls} = 4{\cdot}08 \pm 0{\cdot}7$) (Fig. 3a,b). None the less, the transcript level of DUSP7 was not different among subsets (Fig. 3c).

Discussion

Despite the multi-factorial nature of the RA aetiology, it is currently proposed that CD4 T cells play a major role in the pathogenesis of this disease. Owing to the critical role of phosphorylation levels in regulating intracellular signalling networks that control normal CD4 T cell immune responses, we aimed to compare the expression profile of genes coding for PTPs in cells of healthy volunteers and patients submitted to an EA clinic before being exposed to specific RA therapy.

In this study we have analysed CD4 T cells, which co-operate for B cell activation. It will be also interesting to study the expression profile of PTPs in other cells involved in the pathology, such as B cells. Besides the secretion of autoantibodies, B cells are thought to participate in RA pathogenesis by presenting antigen to T cells and by secreting cytokines [18]. Thus, the proper function of signalling networks regulating these processes should be investigated. Consistent with B cell relevance in RA pathogenesis, an improvement of the clinical symptoms of the pathology

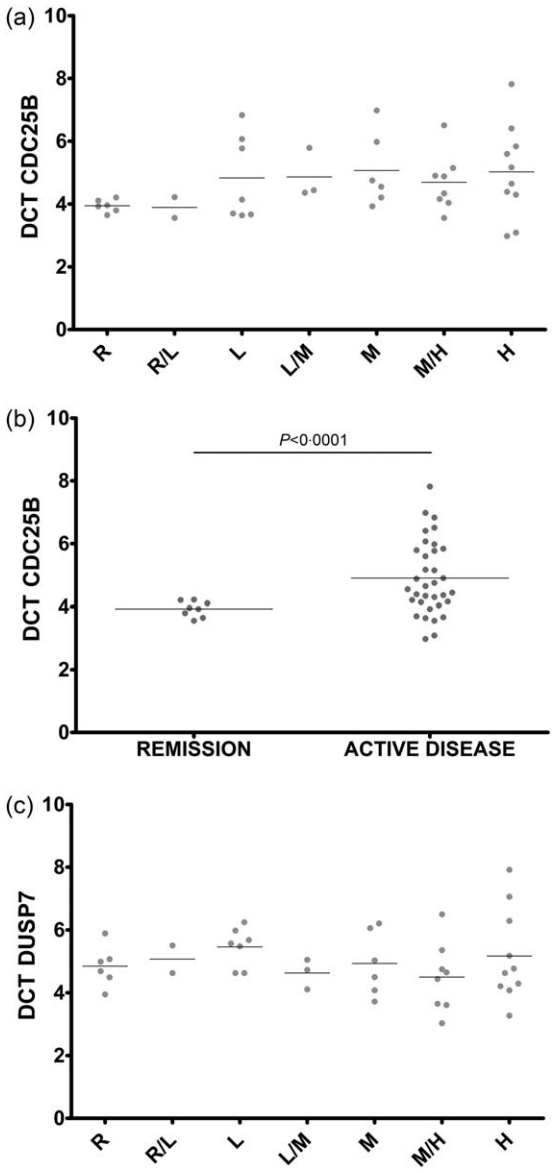

Fig. 3. Low expression of CDC25B in patients with active arthritis. Graphs represent the dCT values of CDC25B (a) and dual-specific phosphatase-7 (DUSP7) (c) in patients in different subsets of disease activity, including: remission (R), remission/low (R/L), low (L), low/moderate (L/M), moderate (M), moderate/high (M/H) and high (H). (b) The graph represents the dCT values of CDC25B in the remission group, resulting from grouping R and R/L subsets, or in the active disease group, resulting from grouping all the remaining subsets. The probability of the Student's *t*-test is indicated.

has been demonstrated by the treatment of patients with rituximab [19], an anti-CD20 antibody, which promotes antibody-dependent cellular cytotoxicity of B cells [20].

The transcript level of DUSP7 was lower in seropositive patients. DUSP7 is a cytoplasmic DSP belonging to the MKPs that regulate the phosphorylation state of the MAPK extracellular-regulated kinase (ERK) [21]. The function of this DSP in T cells is currently not known, and the lower transcript level of DUSP7 in $ACPA^+/RF^+$ patients might indicate a regulatory role in processes deregulated in the seropositive pathology. Association of lymphoid-specific phosphatase (LYP) polymorphism C1858T (R620W) with seropositive RA patients has also been shown [22]. Interestingly, LYP regulates TCR signalling and whether it is also the case for DUSP7 should be investigated. The possible role of DUSP7 as a biomarker of seropositive RA should be assessed further in a larger cohort of patients.

Lower levels of CDC25B were observed in those patients with marked active disease. CDC25B is a known regulator of G2/M transition during the cell cycle [23]. None the less, the function of this molecule in CD4 T cells has not been evaluated. CDC25B is phosphorylated and activated at the centrosome by Aurora A kinase [24], which has been proposed to be required for the dynamics of microtubules supporting intracellular signalling subjacent to the activation of T cells [25]. Thus, it is tempting to speculate that lower levels of CDC25B might promote a higher disease activity by altering T cell sensitivity or activation. The regulatory role of CDC25B during T cell activation should be investigated. The cross-sectional design of our study does not allow determining whether the low level of CDC25B is the origin or the consequence of the inflammatory process. Supporting the latter, we have found that pharmacological activation of PKC and cytosolic Ca^{2+} elevation in CD4 T cells of healthy individuals resulted in down-modulation of CDC25B transcript levels [26]. Whether the treatment of active RA with typical immunosuppressants may restore the expression level of this enzyme should be tested further in a longitudinal follow-up study.

RA pathology has been associated with an anticipated ageing of the immune system. This pathological ageing seems to be due to premature dysfunction of the thymus in RA patients. Early thymus dysfunction promotes an increase in self-replication of T cells promoting an accelerated senescence characterized by short telomeres, cell cycle arrest and secretion of proinflammatory cytokines [27]. This enhanced replicative stress, directed to compensate for the loss of new thymic emigrants to the periphery, renders cells with a low capacity to proliferate. Consistently, the number of T cells in cell cycle in RA patients has been proposed to be lower than in healthy individuals [28]. Interestingly, low levels of CDC25B, which promote cell division, might participate or be a consequence of this process.

Changes in transcript levels might be due to different frequencies of CD4 T cell subtypes in patients. Thus, a deeper analysis of transcript levels in CD4 T cell subpopulations will be needed to understand more clearly the changes found in this work. Importantly, no difference has been found previously in the percentage of peripheral blood naive and memory T cells in RA patients [29]. Although other authors found a reduction in the central memory compartment in RA, the cohort used contained

therapy-submitted patients of long-duration disease [30]. Thus, it is not comparable with the cohort used in this work. In addition, an expansion of effector-memory T cells ($CD4^+CD28^-$) has been broadly found, in particular in patients with severe extra-articular RA [31]. These cells are characterized by a senescent phenotype associated with the expression of NK cell stimulatory molecules, such as NKG2D [32]. We did not find a substantial expansion of $CD4^+CD28^-NKG2D^+$ cells in our cohort, although a slightly higher percentage of this T cell subpopulation was observed in some patients (data not shown). Our finding is consistent with an early stage of the pathology with no signs of severe extra-articular RA.

The most remarkable information in this study was that low transcript levels of CDC25B were associated with subsets of patients with higher disease activity. Whether low expression levels may contribute to the origin or the progression of autoimmune arthritis should be investigated. Lower transcript levels may be the consequence of higher cell activation in a more inflammatory environment in patients with active disease or of recent episodes of enhanced self-replicative stress. Our data suggest that measuring the expression levels of CDC25B might assist in the evaluation of the activity or prognosis of the disease. None the less, a longitudinal study in a larger cohort of patients will be needed in order to assess how patients evolve during the disease or respond to different therapies. This will provide information about the relation between gene expression and the pathogenesis of the RA. We are currently working in this direction.

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Disclosure

The authors declare that there is no financial or commercial conflict of interest regarding the publication of this paper.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Expression of CDC25B and dual-specific phosphatase-7 (DUSP7) in patients classified as rheumatoid arthritis (RA) or undifferentiated arthritis (UA). Graphs represent the dCT value in patients. The horizontal line indicates the average value. The probability of the Student's *t*-test is indicated.