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ORIGINAL ARTICLE

The amount of citrullinated proteins in synovial tissue is related to serum anti-cyclic citrullinated peptide (anti-CCP) antibody levels

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Abstract The objective of this study was to determine the relationship between citrullinated proteins in synovial tissue with peripheral anti-citrullinated peptides autoantibodies (ACPA) and peptidylarginine deiminase (*PADI*) *PADI2*, *PADI3*, and *PADI4* messenger RNA (mRNA) expressions in synovial tissue and fibroblast-like synoviocytes in rheumatoid arthritis (RA) patients. Eleven RA and 12 osteoarthritis (OA) patients who underwent knee replacement surgery were studied. We detected citrullinated proteins in synovial tissue homogenates by western blot and serum ACPA by ELISA to anti-cyclic citrullinated peptide (anti-CCP) antibodies, and *PADI2*, *PADI3*, and *PADI4* mRNA expressions in synovial tissue and in fibroblast-like synoviocytes. Patients with high amount of citrullinated proteins in synovial tissue (3 out of 7) have high levels of anti-CCP in serum. However, in the remaining 4 patients, the amount of synovial citrullinated proteins was minimal and their sera showed low levels of anti-CCP antibodies. Furthermore, we observed an increase in *PADI2* mRNA expression in RA synovial tissue compared with OA patients (p=0.02). We detected *PADI3* mRNA in the synovial tissue of RA patients, but not in the tissue of OA patients. Even though fibroblast-type synoviocytes in RA are not the main source of PADs in the synovial tissue, they express *PADI2* mRNA moderately, *PADI4* mRNA weakly, while there is no detectable expression of *PADI3* mRNA. In conclusion, we found a variety of citrullinated proteins in the synovial tissue of RA patients and the amount of such proteins is related to serum concentration of anti-CCP antibodies. We identified the presence of *PADI3* mRNA expression in synovial tissue and *PADI2* and *PADI4* mRNA expressions in fibroblast-like synoviocytes from patients with RA.

Keywords Anti-citrullinated protein antibodies · Citrullinated proteins · Peptidyl arginine deiminase · Rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease of multifactorial etiology characterized by chronic inflammation of the joints and the presence of anti-citrullinated protein autoantibodies (ACPA) [1]. ACPA are a variety of autoantibodies that recognize different citrullinated proteins and which may be present in serum years before the onset of the rheumatoid disease [2]. Citrullination of proteins is a post-translational modification that causes the conversion of arginine to citrulline. At the protein level, the reaction causes a reduction in the molecular mass of approximately 1.0 Da for each modified arginine. The positive charge of the arginine is lost, so that the isoelectric point is also changed, and interactions with other proteins can be affected [3]. In humans, citrullination is catalyzed by the enzyme peptidylarginine deiminase (PAD), which may exist in five isoforms (PAD1, PAD2, PAD3, PAD4, and PAD6) with differential expression in tissues and organs. Although PAD2 and PAD4 are considered the most important isoforms in the family of RA-related PADs [4], a recent study described a subgroup of patients with RA and interstitial pulmonary disease with anti-PAD3 [5]. It is not yet clear, however, whether these antibodies are specifically against PAD3 or whether they correspond to antibodies with crossed reactivity against PAD4. Few

studies have analyzed the expression of PAD3 in the synovial tissue from RA patients [4].

It is known that inflammatory cells such as monocytes, macrophages [6], neutrophils [7], and T and B cells [8] express PAD2 and PAD4; however, little is known regarding the production of these enzymes by normal or RA fibroblast-like synoviocytes [8], cells that actively participate in the characteristic synovial proliferation observed in RA patients [9].

While the presence of citrullinated proteins is not unique to the inflamed synovium of RA, for they have been detected in other arthropathies [10], the presence of ACPA is highly specific for RA [11]. A great heterogeneity of citrullinated proteins in the synovial tissue of RA patients has been reported [12], and there are several studies that support the idea that these proteins play an important role in its pathogenesis [13, 14]. However, it is unknown whether there is a relationship between the amount of citrullinated proteins in RA synovia and circulating ACPA titers. Therefore, the aim of this study has been to determine the relationship between citrullinated proteins in synovial tissue with peripheral ACPA in patients with RA and with the peptidylarginine deiminases (*PADI*s) messenger RNA (mRNA) in the synovial tissue and fibroblast-like synoviocytes.

Methodology

Patients

We obtained 11 samples of synovial tissue from RA patients (all female), with a mean age of 53 years (range 45–68 years), a mean evolution of 22 years (range 5–33 years), and treated with methotrexate (63 %), prednisone (45 %), hydroxychloroquine (36 %), sulfasalazine (27 %), and leflunomide (27 %). All these patients showed positive anti-cyclic citrullinated peptide (anti-CCP) antibodies, mean 174 U/mL (range 46.4–366 U/mL); 90 % of patients were in remission at the time when samples were taken. We obtained samples of synovial tissue from 12 osteoarthritis (OA) patients (10 females), with a mean age of 77 years (range 68–88 years) and a mean evolution of 15 years (range 3–27 years). All these patients were receiving NSAID and proved negative for anti-CCP antibodies, mean 4.8 U/mL (range 3.8–6.4 U/mL). All synovial biopsies were carried out during knee replacement surgery. Blood samples were obtained from each patient for serological studies. All RA patients met the American College of Rheumatology 1987 revised criteria for RA [15]. The local institutional review board approved this study. All patients gave their written informed consent.

Preparation of synovial tissue samples

Synovial tissue samples were fragmented and homogenized in a lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM DTT, and 1 mM PMSF). Next, the samples were frozen and thawed three times and then centrifuged at 4 °C for 10 min. The supernatants were collected and its protein concentration was determined using bicinchoninic acid method. The supernatants were stored at −20 °C until use.

Cell culture

Synovial tissue was fragmented and incubated in a solution of collagenase type IV (0.025 g/10 mL) (SIGMA, St Louis, MO, USA). Tissue debris was removed and supernatant was centrifuged. The pellet was resuspended in Dulbecco's modified Eagle medium (GIBCO, Life Technologies, Grand Island, NY, USA) with 10 % FBS (GIBCO) and transferred to a culture dish. After four culture passages (approx. 80 % confluence), the CD90 expression [16] was studied by flow cytometry (BD Accuri C6 flow cytometer, Piscataway, NJ, USA) using an anti-CD90 (Thy.1) FITC-conjugated human antibody (eBioscience San Diego, CA, USA).

Isolation of RNA and RT-PCR amplifications

Total RNA was isolated from the synovial tissue using TRIzol reagent (GIBCO) and reverse-transcribed to generate cDNAs using Two-Step quantitative reverse transcription PCR (qRT-PCR; Invitrogen, Carlsbad CA). The *PADI2*, *PADI3*, and *PADI4* mRNA genes were amplified by PCR reactions and normalized to the expression level of the *GAPDH* or *β actin* gene. PCR conditions were as follows: *PADI2* sense 5′ CTCCCCAGACCAGCATACTT 3′; *PADI2* anti-sense 5′ AAGGACCAGGCAAGAGAAGAT 3′; *PADI3* sense 5′ GGTGAACTGTGACCGTGATG 3′; *PADI3* anti-sense 5′ GCTGGAGGTATGGAGGACAA 3′; *PADI4* sense 5′ TGGAACATG GTGCCCTGAG 3′; *PADI4* anti-sense 5′ GGGAGCCACAATATTCACAAGA 3′; *GAPDH* sense 5′ CATGTTCCAATATGATTCCAC 3′ and *GAPDH* anti-sense 5′ CCTGGAAGATGG TGATG 3′; *β actin* sense 5′ GGGTCAGAAGGATTCCTA 3′; *β actin* anti-sense 5′ GGTCTCAAACATGATCTG 3′. PCR amplification of *PADI2*, *PADI3*, *PADI4*, and *β actin* was carried out using the following conditions: 1 cycle 50 °C for 15 min; 1 cycle 95 °C 2 min; and 35 cycles of 95 °C for 15 s and 60 °C for 30 s. For *GAPDH* 1 cycle 50 °C for 15 min; 1 cycle 95 °C for 2 min; and 35 cycles of 95 °C for 15 s and 64 °C for 30 s.

In vitro citrullination

HEp-2 lysate was citrullinated in vitro with rat muscle PAD (SIGMA) using 0.5 U PAD/mg of lysate for 3 h at 55 °C in

buffer containing 0.1 M Tris-HCl, 10 mM $CaCl_2$, and 5 mM DTT, pH 7.4. The reaction was stopped by the addition of 0.5 M EDTA to a final concentration of 20 Mm.

Detection of citrullinated proteins in synovial tissue

The samples were separated by SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and citrullinated proteins were detected by using an anti-modified citrulline (anti-MC) detection kit (Upstate Biotechnology, Temecula, CA). Briefly, citrulline residues of the membranes immobilized proteins were modified by 2, 3 butanedione monoxime and antipyrine in a strong acid solution according to manufacturer's instructions. The modified citrulline residues were then detected with rabbit polyclonal anti-MC antibodies (Upstate Biotechnology) and a goat anti-rabbit IgG-horseradish peroxidase (HRP) antibody conjugate (Upstate Biotechnology) using a chemiluminescent substrate for HRP.

Detection of ACPA

ACPA serum levels were measured using a commercial Quanta Lite CCP3 IgG ELISA (Inova Diagnostics, Inc. San Diego, CA, USA). Results were quantified and the cutoff value (20 U/mL) was set according to the manufacturer's instructions.

Statistical analysis

Mann-Whitney *U* test was used to determine differences between groups. We used GraphPad software version 5.0 (GraphPad software, Inc., La Jolla, CA, USA). We considered *p* values of less than 0.05 to be significant.

Results

Synovium citrullinated protein levels are associated with serum ACPA in RA patients

We asked whether there is a relationship between the amounts of citrullinated proteins in RA synovium and the serum ACPA concentration. Interestingly, we observed a considerable amount of citrullinated proteins in three out of seven (42 %) synovial tissue samples from RA patients (RA1, RA2, and RA7), while in the four remaining ones, only a few could be detected (RA3, RA4–RA6). In contrast, only one band of about 57 kDa could be detected in OA samples (OA1) (Fig. 1). We then analyzed the relationship between the amount of citrullinated proteins in the synovial tissue and anti-CCP antibody serum concentration in each patient. For RA patients, we found that synovial tissue samples with the highest number of bands, corresponding to citrullinated proteins, had the highest concentration of serum antibodies (R-II; >300 U/mL), while samples with little or no presence of citrullinated proteins had a low serum anti-CCP antibodies concentration (R-I; 40–70 U/mL) (Fig. 2). Moreover, in the OA group, all samples were negative for anti-CCP antibodies and the presence of citrullinated proteins was virtually undetectable.

PADI2 and *PADI4* mRNA expression in synovial tissue of RA and OA patients

In order to investigate whether the difference between the amount of citrullinated proteins in the synovial tissue of patients with high serum concentration of anti-CCP antibodies (>300 U/mL) (RA-II: RA1, RA2, RA7) and in the tissue of patients with low concentration of anti-CCP antibodies (40–70 U/mL) (RA-I: RA3–RA6) was due to differences in PAD2 and PAD4 isoform expression, we analyzed *PADI2* and *PADI4*

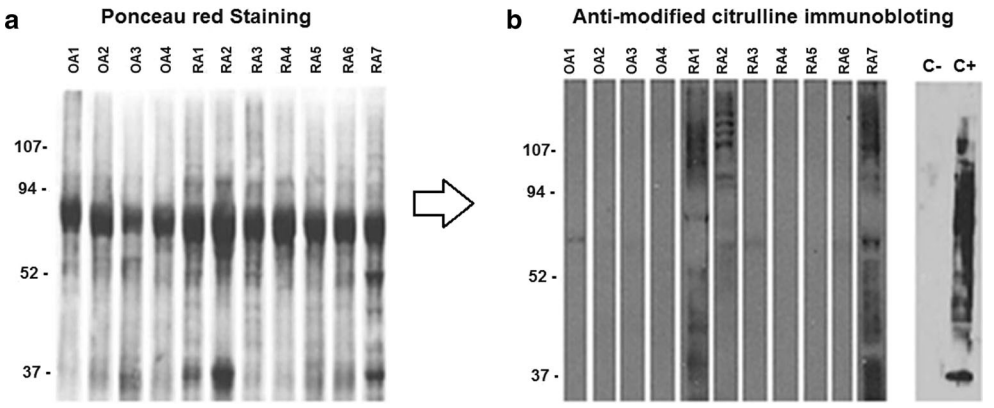

Fig. 1 Different citrullinated proteins profile between synovial tissue samples of RA patients. Proteins in extracts of synovial tissue were separated by SDS-PAGE, transferred to nitrocellulose membranes and stained with Ponceau red to visualize the amount of protein per line (**a**). Anti-MC immunoblotting visualized by chemiluminescence (**b**). Sample synovial tissue from OA (*lines 1–4*) and RA patients (*lines 5–11*). C−, negative control (HEp-2 lysate); C+, positive control (HEp-2 lysate citrullinated in vitro)

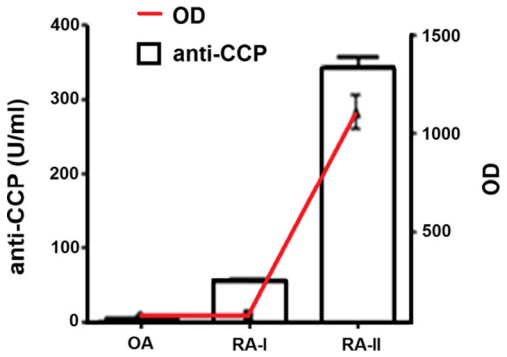

Fig. 2 Association between the amount of citrullinated proteins and anti-CCP antibodies levels. The RA samples were divided according to the amount of citrullinated protein in homogenized synovial tissue; RA-I (RA3–RA6) with little or no citrullinated proteins and RA-II (RA1, RA2, RA7) with high content of citrullinated proteins and OA (OA1–OA4). Densitometry (OD) was used as an indirect measurement of the amount of citrullinated proteins (line). The measurement of anti-CCP antibodies (U/mL) was performed by ELISA (bars). Shown are the mean of every group

mRNA in the synovial tissue, seven RA (RA1-RA7) and eight OA (OA1-OA8). No differences in *PADI2* and *PADI4* mRNA expressions in the synovial tissue of RA-I and RA-II patients were seen in either group (data not show), whereas the *PADI2* mRNA expression was significantly higher in the RA group vs the OA group (p=0.02) (Fig. 3a). However, although the PAD4 isoform has been associated primarily with citrullination in RA, no significant differences in its mRNA were detected between the RA vs the OA groups (Fig. 3b). According to our results, the observed difference between the amounts of citrullinated proteins in the same group of RA was not associated with higher *PADI2* and *PADI4* mRNA expressions in the synovial tissue. Furthermore, possibly due to the small number of samples analyzed, we found no association between the amount of citrullinated proteins in the synovial tissue of RA patients neither with treatment nor with disease duration.

PADI3 mRNA expression in synovial tissue from RA patients

To analyze the *PADI3* mRNA expression, we obtained new samples of the synovial tissue. In three out of four samples from RA patients (RA9–RA11), we detected *PADI3* mRNA, as opposed to the samples from OA patients, in which such isoform was not detectable (OA9–OA12) (Fig. 4). With the same samples, we analyzed the *PADI2* and *PADI4* mRNA expressions. The *PADI2* mRNA expression was similar for both groups, contrasting with the *PADI4* mRNA expression, which was greater in the RA group (Fig. 4).

PADI2, *PADI3*, and *PADI4* mRNA expressions in fibroblast-like synoviocytes from RA patients

Even though fibroblast-like synoviocytes are not the main source of PAD2 and PAD4 in the synovial tissue, we analyzed the *PADI2*, *PADI3*, and *PADI4* mRNA expressions in primary cultures obtained from the samples analyzed under the previous heading to establish what was their contribution in the global expression of such PADs in the synovial tissue. We observed that fibroblast-like synovicites do express *PADI2* mRNA and, apparently, do have a certain contribution in the *PADI2* mRNA expression observed in the synovial tissue. By contrast, the *PADI4* mRNA expression is low and for *PADI3* mRNA undetectable (Fig. 5). We did not observe any differences in the *PAD* mRNA expression between the fibroblast-like synoviocytes of the RA and OA groups.

Discussion

Protein citrullination plays an important role in the generation and maintenance of the RA autoimmune response. There are several antigenic citrullinated proteins that have been related

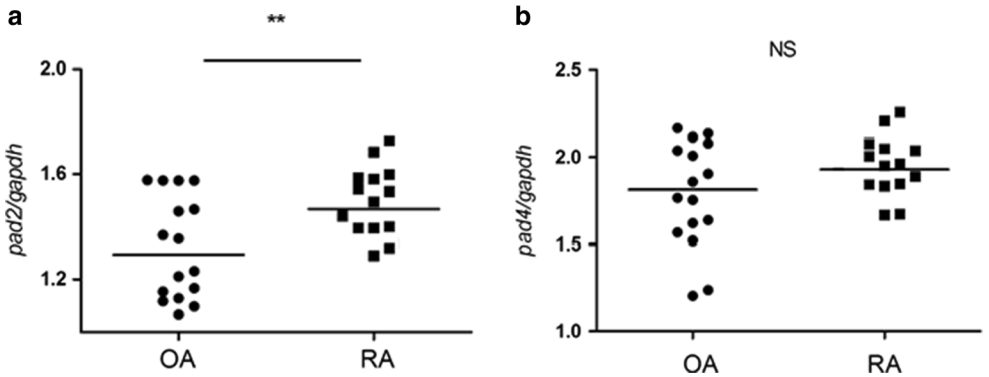

Fig. 3 Relative *PADI2* and *PADI4 mRNA expressions* in the synovial tissue of OA (dots) and RA (squares) patients. RA patients. RNA was isolated from the synovial tissue homogenates and subjected to RT-PCR using specific primers. The data were normalized for *GAPDH* as a reference and the relative *PADI2* (**a**) and *PADI4* (**b**) mRNA expressions are depicted in the graph. Shown are the medians of duplicates. *NS* not significant; **p<0.05 Mann-Whitney *U* test

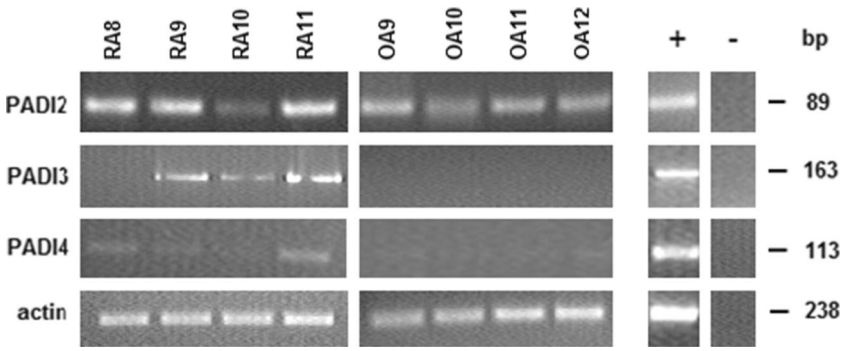

Fig. 4 *PADI3* mRNA expression in the synovial tissue of RA patients. RNA was isolated from synovial tissue homogenates and subjected to RT-PCR using specific primers. The figure depicts representative results of several experiments conducted independently

to RA. These include fibrinogen [17, 18], collagen [19, 20], vimentin [21], and alpha enolase [22]. Herein, we analyzed the presence and amount of citrullinated proteins in synovial tissue samples from RA patients and its possible association with serum ACPA levels, as well as the synovial tissue expression of mRNA for *PADI2* and *PADI4*. We observed a great variability of citrullinated proteins in synovial tissue from RA patients whereas little, if any, citrullination was noted in OA synovial tissue samples. Interestingly, we found a significant relationship between the amounts of synovial tissue citrullinated proteins and circulating anti-CCP antibodies in RA patients; that is to say, the higher the amounts of citrullinated proteins in synovial tissue, the higher the peripheral levels of anti-CCP antibodies. It is worth mentioning, however, that we observed some citrullinated proteins in the synovial tissue with positive (although at low levels) serum anti-CCP antibodies. These results suggest another potential source of citrullinated antigens that allows the generation of anti-CCP antibodies, such as the lungs in smokers [23] or periodontal infection with *Porphyromonas gingivalis* [24–26]. On the other hand, the decrease of citrullinated proteins in synovial tissue has been observed with glucocorticoid therapy, where these drugs altered the expression of these proteins as well as that of PAD2 and PAD4 enzymes resulting in decreased inflammation [27]. Another possible scenario is that of the anti-CCP+ and anti-CCP− patients [28, 29], where it can be postulated a high synovial tissue production of citrullinated proteins in the former and few, if any, in the latter. Unfortunately, we did not include anti-CCP− patients in our study to support this assertion.

We found no association between the amounts of citrullinated proteins with *PADI2* or *PADI4* mRNA expression in synovial tissue of RA patients, despite the fact that there have been reports of a correlation between the degree of synovial inflammation and the expression of PAD2 and PAD4 [27]. In this regard, we do not know if all the RA samples analyzed in that report had the same degree and composition of the cellular infiltrate. If this were the case, we can infer that the difference in the amount of citrullinated proteins in the synovial tissue detected in our patients was due to an increased PAD2 and PAD4 activity, and not to its enhanced expression, for we did not find any differences in their mRNA quantification. Another possible explanation is the presence of the *PADI4* haplotype, initially described in Japanese and Korean populations, which allows higher enzymatic activity [30]. Moreover, this haplotype associated to RA confers higher stability of *PADI4* mRNA. Given this association, it would be interesting to evaluate the *PADI4* haplotype's influence on the enzymatic activity in RA subgroups selected according to the amount of citrullinated proteins in their synovial tissue.

On the other hand, we did confirm the presence of the mRNA of *PADI3* in the synovial tissue of RA patients, which suggests that PAD3 could take part in the generation of antigenic citrullinated proteins in the synovial tissue of RA; PAD3 could even be an antigenic target, as has been previously

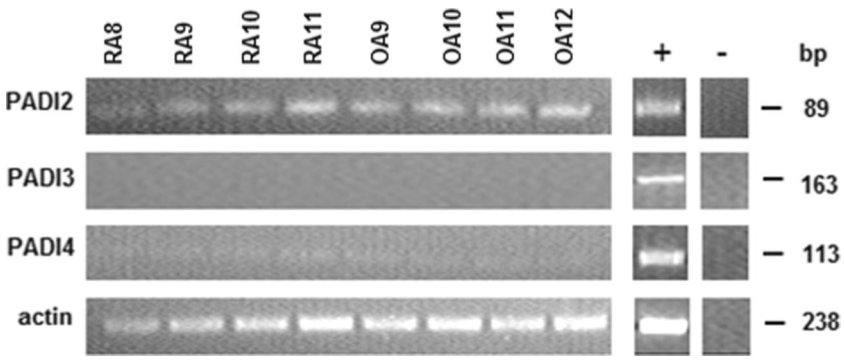

Fig. 5 *PADI2*, *PADI3*, and *PADI4* mRNA expressions in fibroblast-like synoviocytes from RA patients. More than 98 % of cells expressed CD90 after the fourth pass. *PADI2*, *PADI3*, and *PADI4* and β-actin mRNA expressions were measured by RT-PCR. The figure shows representative results of several experiments conducted independently

reported [5]. Although we could show the presence of the mRNA of *PADI3*, the analysis must still be made to ascertain the expression at the protein level.

Chang et al., proposed that there are several features in common between tumor tissues and synovial RA tissue such as angiogenesis, fibrin deposition, defects in cell proliferation, increased coagulant activity, and increased expression of PAD4 [31]. Herein, we observed mRNA of *PADI2* and *PADI4* in cultured RA and OA fibroblast-like synoviocytes. Although the role of PAD4 expression in fibroblast-like synoviocytes in the pathogenesis of RA is still unclear, one possibility is that PAD4 catalyzes the citrullination of histones and in turn modify the expression of genes [32–34]. It is also possible that PAD4 citrullinates fibrin and fibronectin, hence their interaction with growth factor receptors, and consequently, causes abnormal angiogenesis and defective cell proliferation [35]. Further studies are required to elucidate the significance of the expression of PAD2 and PAD4 in RA fibroblast-like synoviocytes.

In summary, we found a great variability of citrullinated proteins in the synovial tissue of RA patients and their amount is related to that of serum anti-CCP antibody concentration. We identified the presence of *PADI3* mRNA expression in the synovial tissue and *PADI2* and *PADI4* mRNA expressions in fibroblast-like synoviocytes from patients with RA.

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Disclosures None.

References

1. Van Venrooij WJ, Pruijn GJ (2000) Citrullination: a small change for a protein with great consequences for rheumatoid arthritis. Arthritis Res 2:249–251
2. Nielen MN, van Schaardenburg D, Reesink HW et al (2004) Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. Arthritis Rheum 50:380–386
3. Tarcsa E, Marecov L, Mei G et al (1996) Protein unfolding by peptidylarginine deiminase. Substrate specificity and structural relationship of the natural substrates trichohyalin and filaggrin. J Biol Chem 271:30709–30716
4. Foulquier C, Sebbag M, Clavel C et al (2007) Peptidyl arginine deiminase type 2 (PAD-2) and PAD-4 but not PAD-1, PAD-3, and PAD-6 are expressed in rheumatoid arthritis synovium in close association with tissue inflammation. Arthritis Rheum 56:3541–3553
5. Giles JT, Darrah E, Danoff S et al (2014) Association of cross-reactive antibodies targeting peptidyl-arginine deiminase 3 and 4 with rheumatoid arthritis-associated interstitial lung disease. PLoS One 9, e98794. doi:10.1371/journal.pone.0098794
6. Vossenaar ER, Radstake TR, van der Heijden A et al (2004) Expression and activity of citrullinating peptidylarginine deiminase enzymes in monocytes and macrophages. Ann Rheum Dis 63:373–381
7. Asaga HK, Kakashima T, Senshu A et al (2001) Immunocytochemical localization of peptidylarginine deiminase in human eosinophils and neutrophils. J Leukocyte Biol 70:46–51
8. Chang X, Yamada R, Suzuki A (2005) Localization of peptidylarginine deiminase 4 (PADI4) and citrullinated protein synovial tissue of rheumatoid arthritis. Rheumatology 44:40–50
9. Neumann E, Lefevre S, Zimmermann B, Gay S et al (2010) Rheumatoid arthritis progression mediated by activated synovial fibroblast. Trends Mol Med 10:458–58
10. Vossenaar ER, Smeets TJ, Kraan MC et al (2004) The presence of citrullinated proteins is not specific for rheumatoid synovial tissue. Arthritis Rheum 50:3485–3494
11. Schellekens GA, Visser H, de Jong BAW et al (2000) The diagnostic properties of rheumatoid arthritis antibodies recognized a cyclic citrullinated peptide. Arthritis Rheum 43:155–163
12. Matsuo K, Xiang Y, Nakamura H et al (2006) Identification of novel citrullinated autoantigens of synovium in rheumatoid arthritis using a proteomic approach. Arthritis Res Ther 8:R175
13. Lundberg K, Nijenhuis S, Vossenaar ER et al (2005) Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. Arthritis Res Ther 7:R458
14. Hill JA, Bell DA, Brintnell W et al (2003) The conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. J Immunol 171:538–541
15. Arnett FC, Edworthy SM, Bloch DA et al (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31:315–324
16. Müller-Ladner U, Ospelt C, Gay S et al (2007) Synovial fibroblasts. Arthritis Res Ther 9:R223
17. Zhao X, Okeke NL, Sharpe O et al (2008) Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. Arthritis Res Ther 10:R94
18. Hermansson M, Artemenko K, Ossipova E et al (2010) MS analysis of rheumatoid arthritic synovial tissue identifies specific citrullination sites on fibrinogen. Proteomics Clin Appl 4:511–518
19. Suzuki A, Yamada R, Ohtake-Yamanaka M et al (2005) Anti-citrullinated collagen type I antibody is a target of autoimmunity in rheumatoid arthritis. Biochem Biophys Res Commun 333:418–426
20. Yoshida M, Tsuji M, Kurosaka D et al (2006) Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. Mod Rheumatol 18:429–441
21. Tilleman K, van Steendam K, Cantaert T et al (2008) Synovial detection and autoantibody reactivity of processed citrullinated isoforms of vimentin in inflammatory arthritides. Rheumatology 47:597–604
22. Kinloch A, Tatzer V, Wait R et al (2005) Identification of citrullinated alpha-enolase as a candidate autoantigen in rheumatoid arthritis. Arthritis Res Ther 7:R1421
23. Klareskog L, Stolt P, Lundberg K et al (2006) A new model for an etiology of rheumatoid arthritis: smoking may trigger HLA-DR (shared epitope)-restricted immune reactions to autoantigens modified by citrullination. Arthritis Rheum 54:38–46
24. Marchant C, Smith M, Proudman S et al (2013) Effect of porphyromonas gingivalis on citrullination of proteins by macrophages in vitro. J Periodontol 84:1272–1280
25. Rutger PG (2012) Rheumatoid arthritis and periodontitis-inflammatory and infectious connections. J Oral Microbiol 4:11829

26. Bongartz T, Cantaert T, Atkins SR et al (2007) Citrullination in extra-articular manifestations of rheumatoid arthritis. Rheumatology 46:70–75
27. Makrygiannakis D, Shankar R, Engströnm M et al (2012) Local administration of glucocorticoids decreased synovial citrullination in rheumatoid arthritis. Arthritis Res Ther 14:R20
28. Daha NA, Toes RE (2011) Rheumatoid arthritis: are ACPA-positive and ACPA-negative RA the same disease? Nat Rev Rheumatol 7: 202–203
29. Ohmura K, Terao C, Maruya E et al (2010) Anti-citrullinated peptide antibody negative RA is a genetically distinct subset: a definitive study using only bone erosive ACPA negative rheumatoid arthritis. Rheumatology 49:2298–2304
30. Suzuki A, Yamada R, Chang X et al (2003) Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genetics 34:395–402
31. Chang X, Han J (2006) Expression of peptidylarginine deiminase type 4 (PAD4) in various tumors. Mol Carcinog 45:183–196
32. Wang Y, Wysocka J, Sayegh J et al (2004) Human PAD4 regulates histone arginine methylation levels via demethylimination. Science 306:279–283
33. Dong S, Zhang Z, Takahara H (2007) Estrogen-enhanced peptidylarginine deiminase type IV gene (PADI4) expression in MCF-7 cells is mediated by estrogen receptor-α-promoted transfactors activator protein-1, nuclear factor-Y and Sp1. Mol Edocrinol 21:1617–1629
34. Nakashima K, Arai S, Nariai Y et al (2013) PAD4 regulates proliferation of multipotent haematopoietic cells by controlling c-myc expression. Nat Commun 4:1836
35. Chang X, Yamada R, Suzuki A et al (2005) Citrullination of fibronectin in rheumatoid arthritis synovial tissue. Rheumatology 44: 1374–1382