14-3-3η: a novel biomarker platform for rheumatoid arthritis

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ABSTRACT

14-3-3 proteins are a conserved family of 7 isoforms with diverse cellular functions found predominantly intracellularly. The 14-3-3η isoform is expressed extracellularly in the joints of patients with rheumatoid arthritis (RA) and expression in both serum and joint fluid correlates strongly with expression of metalloproteinases. 14-3-3η activates proinflammatory signalling cascades and inflammatory mediators relevant to the pathogenesis of RA. A new ELISA based assay has diagnostic utility for RA with sensitivity of 63.6% and specificity of 92.6% using the optimal cut-off from ROC analysis of 0.19ng/ml. Adding 14-3-3η to anti-cyclic citrullinated peptide antibodies (ACPA) resulted in an identification rate of 72% compared to 59% for ACPA alone. Adding rheumatoid factor (RF) to ACPA increased diagnostic capture from 59% to 72% and this increased further to 78% when 14-3-3η was added. Positive 14-3-3η status is also significantly associated with radiographic progression in early RA at years 1, 3 and 5 indicating prognostic utility. Extracellular 14-3-3η elicits the production of autoantibodies to the native protein, which also possess diagnostic utility. These do not correlate with expression of the protein and have complementary diagnostic utility. The presence of either the protein or its autoantibodies is observed in 90% of patients with early RA. Together with RF and/or ACPA this may result in identification of 95% of patients with early RA.

Introduction

The benchmark for the management of rheumatoid arthritis (RA) has been raised towards early identification and intervention aimed at halting evolution from arthralgia to overt RA (1, 2). In particular, primary care physicians require simple 'red-flag' tools which indicate that a patient presenting with arthralgia ought to be referred for further evaluation to a rheumatologist. Impressive data for prediction algorithms have been developed based on a combination of clinical and laboratory parameters, such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA) (3-6). However, the former lack feasibility in primary care due to their complexity, while laboratory parameters alone lack sufficient predictive capacity. There is therefore a major unmet need for the development and validation of new biomarkers that have predictive capacity for RA. A second major unmet need is the assessment of risk for structural progression in RA, particularly in early disease when the implementation of personalised treatment strategies are aimed at achieving early remission and prevention of structural damage (7). Data from the Leiden early arthritis cohort have shown that currently available clinical and laboratory parameters contribute only 32% to the explained variance of structural damage progression (8). The 14-3-3η biomarker platform is a recent discovery with the potential to significantly address both of these major unmet needs in the field.

14-3-3 Proteins

14-3-3 proteins represent a family of ubiquitously expressed intracellular chaperonins that are exclusively expressed in eukaryotic cells. The family consists of seven isoforms and share more than 50% amino acid homology between them: beta (β), epsilon (ε), gamma (γ), eta (η), tau (τ), zeta (ζ), and sigma (σ).

These proteins either homo- or heterodimerise through their N-terminus to form an "amphipathic groove" that allows them to interact with more than 200 intracellular proteins (9). Through these intracellular protein-protein interactions, the 14-3-3 family is involved in coordinating an array of biological processes including protein trafficking, cellular signalling, and cytoskeletal transport (10).

Extracellular expression of 14-3-3η, as is the case of RA, is believed to be mediated in part through an exosomal process as 14-3-3 proteins have been described as a key component of exosomes (11) released by activated immune cells including dendritic cells, T-cells, B-cells, macrophages as well as epithelial cells suggesting that externalisation of 14-3-3 proteins is the result of an active secretory mechanism in inflammation.

Discovery of 14-3-3η in arthritis

The study of the externalisation of 14-3-3σ (one of the other 14-3-3 family members) in skin fibroblasts and its association with the up-regulation of metalloproteinase (MMP) expression was the basis for the first analysis of 14-3-3 proteins in patients with RA (12).

Immunoblot experiments of both the synovial fluid and serum of patients with inflammatory arthritis using keratinocyte lysate as a positive control revealed the presence of two isoforms of 14-3-3, γ and η, with the latter being the predominant isoform based on mass spectrometry data (12). Levels of 14-3-3η were up to 5 times higher in synovial fluid than in matched serum, indicating that the synovium is the likely the source of this biomarker. Serum 14-3-3η expression levels in arthritis were significantly higher than in healthy controls. Both serum and synovial levels of 14-3-3η correlated strongly with levels of MMP1 and MMP3 highlighting a possible relationship between 14-3-3η and joint damage.

Diagnostic utility of 14-3-3η in RA

An ELISA for 14-3-3η has been developed and validated according to the performance criteria proposed by the OMERACT Soluble Biomarker Working Group (7). Serum levels of this protein do not vary according to age, gender, maintenance at room temperature, several freeze-thaw cycles, or potential interfering factors in peripheral blood including RF (13).

The specificity of serum 14-3-3η for RA was assessed in 619 subjects including healthy and disease controls, similar to assessments of anti-CCP for registration studies (13). The results demonstrate that median 14-3-3η concentrations are significantly higher in RA patients with established disease *versus* healthy individuals (1.12 *vs*. 0.00 ng/mL) and controls, which included other arthropathies, connective tissue disorders, and autoimmune diseases (0.02 ng/mL). Receiver operator characteristic (ROC) curve analysis comparing established RA to healthy subjects demonstrated a significant area under the curve (AUC) of 0.89 (95% CI:0.85–0.93) with an optimal cut-off of ≥0.19 ng/mL yielding 77.0% sensitivity, 92.6% specificity, a likelihood ratio (LR) positive of 10.4, a PPV of 0.70 and an NPV of 0.80.

Since early diagnosis coupled with an effective treatment strategy is key to improving outcomes in RA, the diagnostic utility of serum 14-3-3η was also assessed in an early RA cohort with median disease duration of <3.5 months. Median 14-3-3η levels in early RA were significantly higher than in healthy individuals and disease relevant controls [0.76 *vs*. 0.02 ng/mL]. At a cut-off of ≥0.19 ng/mL, the ROC curve yielded a sensitivity of 63.6%, a specificity of 92.6%, LR positive of 8.6, a PPV of 0.57 and an NPV of 0.78.

As ACPA and RF are often used together to inform an RA diagnosis, the incremental benefit of adding 14-3-3η to each of the markers was assessed. Adding 14-3-3η to ACPA resulted in an identification rate of 72% compared to 59% for ACPA alone. Adding RF to ACPA increased diagnostic capture from 59% to 72% and this increased to 78% when 14-3-3η was added.

Pathogenic role of 14-3-3η in RA

Certain biomarkers exist as "bystanders" whereas others are intimately linked to the pathogenesis of the disease. To determine if 14-3-3η is involved in perpetuating disease, *in vitro* cell stimulation studies were performed using human recombinant 14-3-3η at concentrations that reflect levels in RA patient serum. Cell stimulation studies revealed that 14-3-3η preferentially stimulates cells of the innate immune system, leading to the activation of key signalling cascades such as MAPK/ERK, SAPK/JNK and the JAK-STAT pathway that regulate the production of inflammatory and degradative factors (Fig. 1). Notable differences between 14-3-3η and TNF-α were that 14-3-3η did not activate p38MAPK or the NF-κB pathway. Several RA relevant transcripts were shown to be upregulated by 14-3-3η and included pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, TNF-α, and joint degradation factors such as MMP-9 and receptor activator of nuclear factor kappa-B ligand (RANKL) (14). Early data indicates that these joint damage effects can be blocked by pre-incubation with 14-3-3η antibodies. Studies are currently underway to assess the therapeutic potential of targeting 14-3-3η *in vivo* using the collagen-induced arthritis model.

The 14-3-3η biomarker platform

As noted above, 14-3-3η is normally an intracellular protein. When it is externalised, it is viewed by the immune system as foreign and there, it also encounters peptidyl arginine deiminases (PAD) enzymes which results in its citrullination. Therefore, there are two forms of 14-3-3η auto-antibodies circulating in the serum of RA patients, those that react with the native form of the protein and those that specifically recognise the citrullinated form. The 14-3-3η Biomarker Platform (Table I) comprises four (4) distinct yet related biomarkers which are currently being investigated, alone and in combination, for their clinical utility in RA and for joint involvement risk in patients with autoimmune diseases.

Prognostic utility of 14-3-3η in RA

An association between the levels of 14-3-3η and MMPs has been previously reported (12). *In vitro* results, specifically the induction of MMPs and RANKL, support a role for 14-3-3η in the joint damage process. Preliminary data on the prognostic utility of the 14-3-3η protein was derived from an analysis of 33 patients with RA according to the 1987 ACR classification criteria, which recruited patients with synovitis affecting at least 3 joints for 1–12 months (15). Median 14-3-3η levels were significantly higher in early

Table I. The 14-3-3η Biomarker Platform

Biomarker	Measures
14-3-3η Protein	Levels of protein in serum (or plasma)
14-3-3η Citrullinated protein	Burden of citrullination in relation to total protein
Anti-14-3-3η Auto-antibodies	Levels of auto-abs in serum (or plasma)
Anti-cit 14-3-3η Auto-antibodies	Ratio of auto-abs reacting to citrullination sites

Table II. Sensitivity, specificity, and likelihood ratios for RF, ACPA, 14-3-3η, and RF and/or ACPA positivity with and without inclusion of 14-3-3η (13).

	Sn/Sp	LR+/LR-
Early RA *vs*. healthy controls		
RF	0.57/0.85	3.8/0.51
CCP	0.59/0.99	59.00/0.41
14-3-3η	0.63/0.93	9.00/0.40
RF and/or ACPA	0.71/0.84	4.44/0.35
RF and/or ACPA and/or 14-3-3η	0.78/0.78	3.55/0.28
Early RA *vs*. all controls		
RF	0.57/0.84	3.56/0.51
CCP	0.59/0.98	29.50/0.42
14-3-3η	0.63/0.86	4.50/0.43
RF and/or ACPA	0.71/0.83	4.18/0.35
RF and/or ACPA and/or 14-3-3η	0.78/0.74	3.00/0.30
Established RA *vs*. healthy controls		
RF	0.84/0.85	5.60/0.19
CCP	0.79/0.99	79.00/0.21
14-3-3η	0.77/0.93	11.00/0.25
RF and/or ACPA	0.88/0.84	5.50/0.14
RF and/or ACPA and/or 14-3-3η	0.90/0.78	4.09/0.13
Established RA *vs*. all controls		
RF	0.84/0.84	5.25/0.19
CCP	0.79/0.98	39.50/0.21
14-3-3η	0.77/0.86	5.50/0.27
RF and/or ACPA	0.88/0.83	5.18/0.14
RF and/or ACPA and/or 14-3-3η	0.90/0.74	3.46/0.14

RA: rheumatoid arthritis; RF: rheumatoid factor; ACPA: anti-citrullinated protein antibody; Sn: sensitivity; Sp: specificity; LR+: positive likelihood ratio; LR-: negative likelihood ratio.

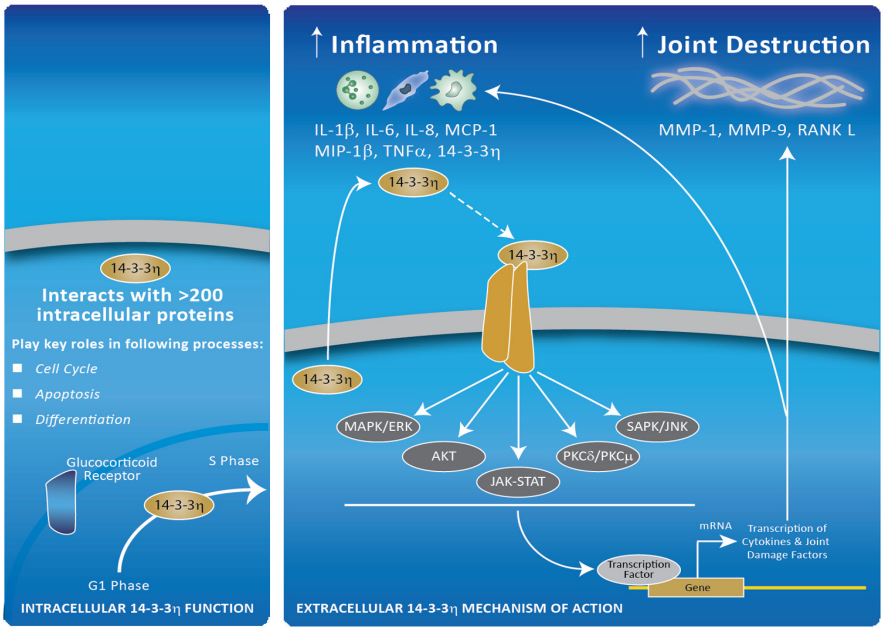

Fig. I. The 14-3-3η Signalling Map

RA patients with radiographic progression *versus* those who did not progress [2.68 ng/ml *vs*. 0.09 ng/ml, p=0.006]. Using the diagnostic cut-off of ≥0.19 ng/ml the odds of radiographic damage progression in early RA was 6.2, which increased to 10.2 with a cut-off of ≥0.40 ng/ml and 0.80 ng/ml. These clinical observations align with 14-3-3η's mechanistic role as a potent, dose-dependent upregulator of factors that perpetuate joint damage. When 14-3-3η positive status was combined with other significant variables (DAS28, RF) into a multivariate model, it was shown to contribute to an increase in the total variance for radiographic progression.

Analysis of results from two additional cohorts support the prognostic utility of 14-3-3η in RA. In one cohort, 148 patients with arthralgia who were either RF or ACPA positive were recruited (17). In the second cohort, 409 patients with early RA and meeting the ACR-EULAR classification criteria for early RA were recruited (18). The contribution of 14-3-3η was assessed by multivariate analysis to determine whether it was associated with development of RA in the arthralgia cohort or radiographic changes in the early RA cohort as assessed using the Sharp-van der Heijde score (SHS). Median 14-3-3η levels were significantly higher in the arthralgia group that developed RA. 14-3-3η and ACPA titres, but not titres of RF, were associated with the development of RA with LRs of 4.12 and 4.16 (17). In the early RA cohort, 67% of patients were 14-3-3η positive and levels of 14-3-3η and RF, but not ACPA, were significantly higher in patients who progressed radiographically by year 5 (18). At baseline, 14-3-3η positive patients had significantly higher joint damage progression over 5 years than the 14-3-3η negative patients (SHS change in 3.0 *vs*. 1.5). Positive 14-3-3η status was significantly associated with radiographic progression at years 1, 3 and 5 with LR ranging from 3.8 to 6.8. In a multivariate model, independent predictors of progression over 3 years were disease duration (LR=8.3) and 14-3-3η titres (LR=6.2).

Theragnostic utility of 14-3-3η in RA

We and others have reported on an interplay that exists between 14-3-3η and TNF-α and more specifically that 14-3-3η can induce various inflammatory factors including IL-6 in a dose-dependent fashion (14). Results from three independent investigational studies indicate that patients with lower baseline 14-3-3η levels have a higher likelihood (LR) of achieving better clinical outcomes irrespective of the type of therapy that is administered. In a cohort of 409 early RA patients, baseline 14-3-3η levels ≤0.40 and ≤0.80 ng/ml were associated with a higher likelihood of achieving DAS remission to standard DMARDs (DAS-ESR <2.6) (19). Similar observations were seen in two established RA studies, 41 Japanese patients who were treated with tocilizumab and 74 Canadian patients treated with various anti-TNFs (20, 21). Based on these clinical data, taken together with 14-3-3η's biological mechanism, it is postulated that with higher concentrations of circulating 14-3-3η there is a lower likelihood of response to therapy as 14-3-3η may perpetuate the "cytokine storm". Efforts are underway to understand the interplay between 14-3-3η and various chemokines/cytokines relevant to RA, and how specific therapies impact 14-3-3η serum levels.

Anti-14-3-3η auto-antibodies

As noted above, extracellular 14-3-3η is seen by the immune system as foreign, which results in the development of two forms of auto-antibodies: those that recognise the native form (14-3-3η pan-AAb) and a second that recognise citrullinated 14-3-3η. Recent methods and data have been presented on the anti-14-3-3η auto-antibodies wherein serum or plasma levels were measured on the MSD electrochemiluminescent platform using 6 prioritised peptides with a diagnostic cut-off of ≥380U/ml (22). The ROC curve for early RA *versus* healthy and disease relevant controls yielded a sensitivity of 73%, a specificity of 79%, a likelihood ratio (LR) positive of 3.47, a PPV of 0.61 and an NPV of 0.86. Unlike the 14-3-3η protein, no significant correlation was observed with RF or ACPA. In addition, no significant correlation was observed between titres of 14-3-3η protein and its auto-antibodies, resulting in their combined expression identifying >90% of early RA patients and the presence of any one of RF, ACPA, 14-3-3η protein, or anti-14-3-3η autoantibodies identifying 95% of patients. Of particular interest, 14-3-3η antibodies recognise epitopes within and around the ligand-binding amphipathic groove of 14-3-3η suggesting that there could be functional consequences of their binding. While this data supports the complementary aspects of the 14-3-3η biomarker platform with existing biomarkers used in RA, it is important to point out that 14-3-3η, like all biomarkers assessed in RA, is not a "gold standard" for diagnosis in all individual patients such as haemoglobin A1c for diabetes. Moreover, further clinical validation according to the OMERACT framework (7) is necessary before the 14-3-3η biomarkers attain the status of a monitoring marker such as serum cholesterol.

Future directions

Several multi-centre independent studies are underway to expand our understanding of the diagnostic, prognostic and theragnostic applications of 14-3-3η biomarkers to assist with RA patient management. Their mechanistic role in the joint damage process, together with positive signals in cohorts of patients with erosive disease, supports 14-3-3η investigation as an early indicator of joint involvement risk in patients with autoimmune diseases.

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