Assessing RA-patient Autoantibody Response to Citrullinated Immunome Proteins

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Technology background

Recombinant protein expression is a fundamental technique that underpins clinical diagnostics, drug discovery and screening, vaccine development and pure research for elucidating mechanisms of disease development and progression. However, high-throughput production of correctly folded and functional, full-length human proteins has a very high failure rate. Protein folding is a highly complex process requiring a combination of an aqueous environment, chaperones, post-translational modifications and the formation of multimeric structures held together by covalent bonds. Any deviation from the correct sequence of events can result in a misfolded protein. Loss of protein function is directly linked to misfolding. Use of misfolded proteins in downstream assays and interactions studies can result in identification of false positive biomarkers.

The Sengenics KREX technology utilises the biotin carboxyl carrier protein (BCCP) as a folding marker and solubility enhancer which results in consistent, high-throughput expression of full-length, correctly folded and functional proteins. BCCP-protein fusions are capable of being biotinylated either *in vivo* or *in vitro*, allowing the use of highly specific biotin-streptavidin interaction for surface capture. As biotinylated proteins bound to a streptavidin-coated surface show negligible dissociation, this interaction provides a vastly superior means for tethering proteins to a planar surface and is ideal for applications such as protein microarrays, glass micro-titer plates, SPR and bead-based assays.
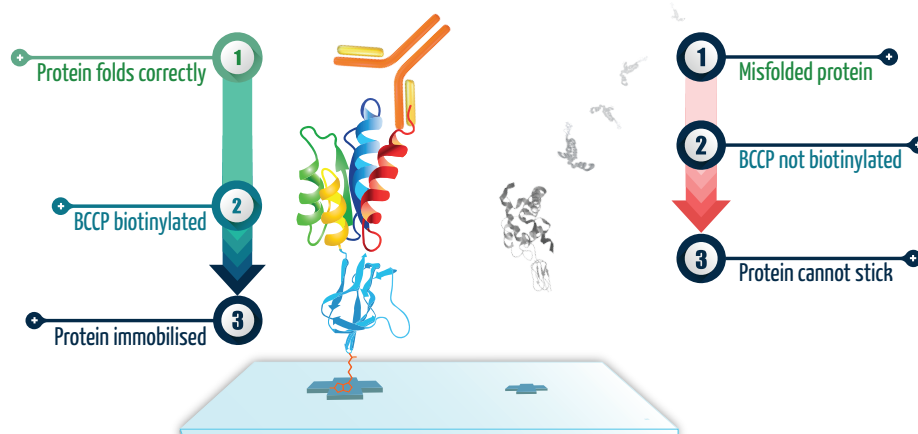

Figure 1: When the protein of interest is correctly folded, it co-translationally drives the correct folding of BCCP, which then becomes biotinylated and allows the fusion protein to become attached to the surface. On the other hand, when the protein of interest is grossly misfolded, it co-translationally drives misfolding of BCCP so it can no longer become biotinylated, preventing it from attaching to the surface. These proteins simply get washed away.

Introduction

In addition to producing antibodies against foreign molecules, the immune system generates antibodies to self-antigens ("autoantibodies") in response to many pathological processes. It is believed that autoantibodies are generated through over-expression, mutation, release of proteins from damaged tissues, mis-folding or mis-presentation of proteins which leads to their recognition by the immune system. Unlike other serological targets, autoantibodies are stable, highly specific, easily purified from serum and are readily detectable with well-validated secondary reagents. Due to their inherent amplification within the immune system, autoantibodies are relatively abundant and are easily measured at exceptional sensitivity using the KREX platform, making them ideal for early diagnosis of disease.

Citrullination is a post-translational modification of arginine side chains catalysed by peptidylarginine deiminase (PAD) enzymes. This induces a change in the protein structure, antigenicity and function. Leveraging the KREX technology, we have developed a fully quantitative protein array platform, which enables the simultaneous screening of thousands of proteins towards autoantibody responses to different diseases. As all the proteins on the surface are correctly folded and functional, post-translational modifications, such as citrullination, of these proteins are possible. This results in a highly robust method for autoantibody biomarker discovery, specifically in diseases such as Rheumatoid Arthritis, Lupus, Multiple Sclerosis and Alzheimer's.

Citrullination Case Study

Study Design:

The overall objective of the study was to identify known and novel autoantibody responses to citrullinated proteins on the Immunome protein array using Rheumatoid Arthritis (RA) patient serum samples. The first objective was to enzymatically convert protein surface arginine to citrulline groups on the Sengenics Immunome protein array. Peptidylarginine deiminase (PAD) 2 and 4 were incubated on the protein array for the enzymatic conversion of arginine to citrulline groups. The Sengenics Immunome protein array was then incubated with anti-citrulline antibody and fluorescently-labelled detection antibody to detect citrulline groups. The second objective of the study was to identify known and novel autoantibody responses of pooled sera from RA patients and pooled sera from matched healthy controls on the citrullinated protein array to identify signature autoantibodies specific to RA. For this assay, protein arrays were incubated with either PAD2 or PAD4, and subsequently incubated with pooled RA patient sera and pooled matched healthy control sera to capture and identify RA-specific autoantibodies.

Study Objective 1: *Detection of citrullinated proteins on the Sengenics Immunome protein array*

Results:

The results indicate that 1131/1622 (70%) and 1183/1622 (73%) of the Immunome proteins were citrullinated by PAD4 and PAD2, respectively, showing significantly higher intensities when compared to the non-modified Immunome proteins. The analysis also showed 708 citrullinated proteins overlap between the 2 enzyme types.

PAD4 Citrullinated Proteins	
NCK1	HUMAN Cytoplasmic protein NCK1
PRKD3	HUMAN Serine/threonine-protein kinase D3
PIM2	HUMAN Serine/threonine-protein kinase pim-2
AKT1	HUMAN RAC-alpha serine/threonine-protein kinase
IST1	HUMAN IST1 homolog
FSCN1	HUMAN Fascin
TMEM108	HUMAN Transmembrane protein 108
TP73	HUMAN Tumour protein p73
PXN	HUMAN Paxillin
NCK1	HUMAN WNT1-inducible-signaling pathway protein 3

Table 1: List of 10 out of the 1131 significant citrullinated proteins by PAD4 enzyme on the Sengenics Immunome protein array

PAD2 Citrullinated Proteins	
PRKD3	HUMAN Serine/threonine-protein kinase D3
SSB	HUMAN Lupus La protein
AKT1	HUMAN RAC-alpha serine/threonine-protein kinase
OGG1	HUMAN N-glycosylase/DNA lyase
VIM	HUMAN Vimentin
PIM2	HUMAN Serine/threonine-protein kinase pim-2
NCK1	HUMAN Cytoplasmic protein NCK1
HOXA9	HUMAN Homeobox protein Hox-A9
HNRNPA2B1	HUMAN Heterogeneous nuclear ribonucleoproteins A2/B1
PKM	HUMAN Pyruvate kinase PKM

Table 2: List of 10 out of the 1183 significant citrullinated proteins by PAD2 enzyme on the Sengenics Immunome protein array

Study Objective 2: *Identify known and novel autoantibody biomarkers against citrullinated proteins in RA*

Results:

Citrullination with PAD2: After confirming protein citrullination, pooled RA patient sera and pooled matched healthy control sera were screened against PAD2 treated Immunome protein arrays. Differential analysis between the 2 cohorts identified elevated autoantibody responses towards known RA-associated citrullinated proteins such as: SSB, VIM and HRNRPA2B1 (Figure 3).
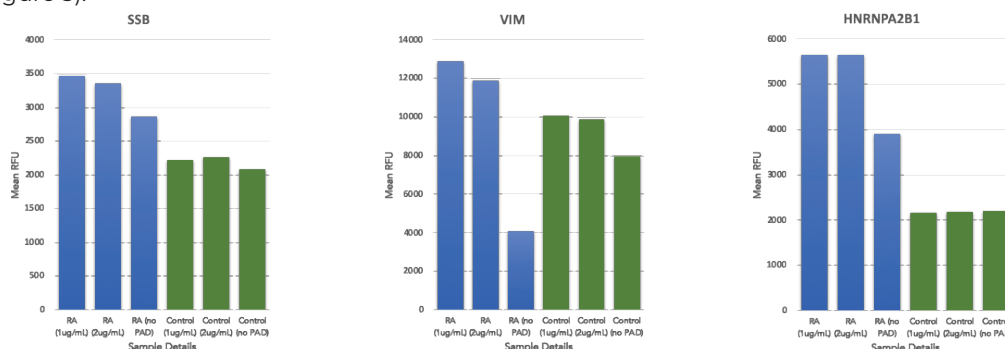

Figure 3: Autoantibody profiles for SSB, VIM and HRNRPA2B1 across all pooled RA (represented in blue) and pooled healthy sera (represented in green) with varying concentrations of PAD2, from left to right: 1μg/ml, 2μg/ml and 0μg/ml. The y-axis represents the average relative fluorescent unit (RFU) between the 4 replica spots of each protein across the different samples.

Citrullination with PAD4: After confirming protein citrullination, pooled RA patient sera and pooled matched healthy control sera were screened against PAD4 treated Immunome protein arrays. Differential analysis of the 2 cohorts identified potential novel autoantibody biomarkers towards citrullinated proteins in the pooled RA patients sera that were not detected on the non-citrullinated protein arrays ("no PAD") (Figure 4).
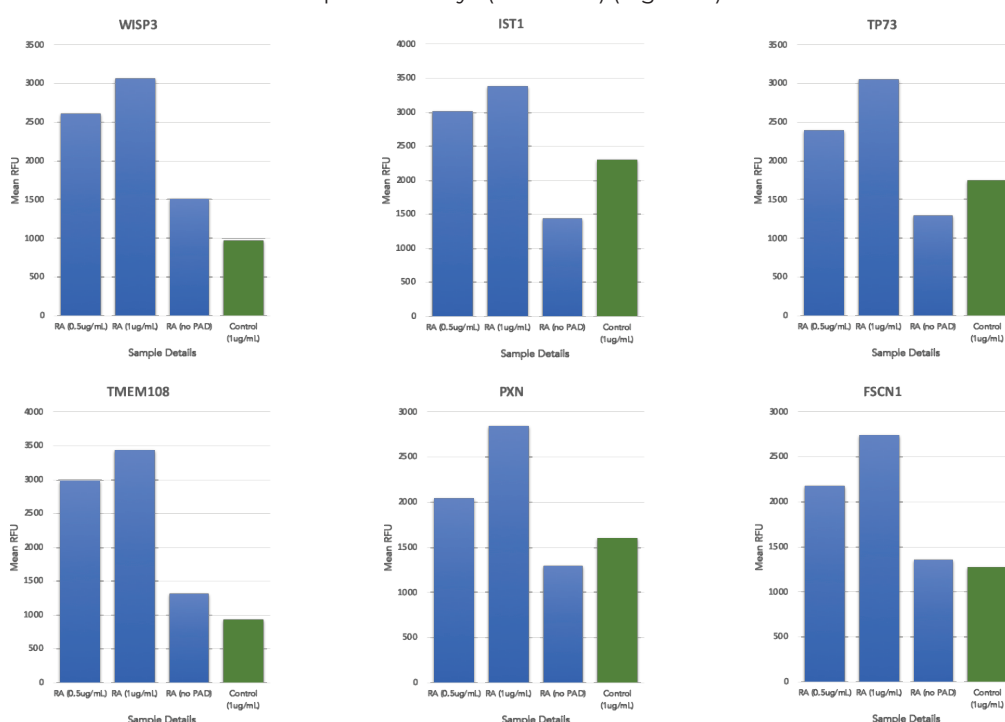

Figure 4: Autoantibody profiles against 6 of the novel citrullinated antigens (WISP3, IST1, TP73, TMEM108, PXN and FSCN1) across all pooled RA (represented in blue) and pooled healthy sera (represented in green) with varying concentrations of PAD4, for pooled RA sera, from left to right: 0.5μg/ml, 1μg/ml and 0μg/ml and for pooled healthy control sera: 1μg/ml. The y-axis represents the average relative fluorescent unit (RFU) between the 4 replica spots of each protein across the different samples.

Conclusion:

The ability to post-translationally modify native proteins to citrullinated proteins using PAD2 and PAD4 enzymes has been successfully demonstrated using the Sengenics Immunome protein array platform. The current study represents the largest investigation of citrullinated proteins (with n > 1000 proteins) for the quantification of autoantibody repertoires in human sera, specifically, exploring sera collected from RA patients. This is highly beneficial considering the role of post-translationally modified proteins (e.g. citrullination, phosphorylation, oxidation etc) in a wide spectrum of diseases.