Identification and Characterization of Novel Autoantibody Biomarkers for Rheumatoid Factor-Negative and Accp- Negative Rheumatoid Arthritis

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Abstract – **Rheumatoid arthritis (RA) is an inflammatory autoimmune disease in which 30% of patients are seronegative for the two serological RA markers, rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACCP). However, both markers also have lower sensitivities in early stages of the disease. The lack of effective biomarkers for this subpopulation of RA patients causes a delayed diagnosis. Therefore, discovery of novel circulating autoantibody biomarkers for these early RF-negative and ACCP-negative RA patients are advance planning of these subsets. 136 enriched antigenic markers were identified by high-throughput screening an RA library with autoantibodies in the sera of RA patients (positive selection) and healthy control (negative selection). Phage-ELISA-based re-screening determined 13 out of 136 enriched antigenic targets in which increased antibody levels rised up. Two of the 13 identified targets, namely special A-T rich DNA binding protein (SATB1) and EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1), which had highest increased antibody levels, were selected for further characterization. Reactivity of serum antibodies against these proteins was tested in a protein ELISA using the sera of 137 RA patients and 159 healthy controls. Autoantibodies against SATB1 were detected with 79% sensitivity and 82% specificity, while 87% sensitivity and 52% specificity were obatained for antibodies directed against EFEMP1. With regard to cytokine assay, interestingly, pro-inflammationary cytokines including TNF (tumour necrosis factor) increased over 1000 pg/ml or IL-4 (interleukin-4) or IL-13 similarly increased up to 90 pg/ml and 102 pg/ml, respectively for co-culture with anti-SATB1 monoclonal antibody. Under treatment with EFEMP1 mAb resulting IL-1, TNF and IL-4 triggered up to 1300 pg/ml, 1250 pg/ml and 180 pg/ml, respectively. In T cell phenotype characterization, two subsets of $CD4^+$T cell and $CD8^+$ cell were stimulated and differentiated from peripheral blood mononuclear cells (PBMCs) at 29.7% and 1.2% for anti-SATB mAb; 31.3% and 2.8% for anti-EFEMP1 mAb.**

Keywords – **SATB1, EFEMP1, biomarkers, rheumatoid arthritis, phage display.**

I. Introduction (Briefly)

The presence of autoantibodies is characteristic of rheumatoid arthritis (RA), in which they serve as biomarker for diagnostics and therapeutic development. These humoral biosignatures are usually taken as a sign that autoreactivity is driving the disease. Moreover, an imbalance of pro-inflammatory and anti-inflammatory cytokines, autoreative and inflammatory T helper 1 (Th1) cells, regulatory and suppressive T cells results in the loss immune tolerance, the breakdown of immune homeostasis and the subsequent appearance of exacerbated autoreactive B cells that form autoantibodies (**Gonzalez-Rey E et al., 2007).** Theories on the pathogenesis of RA have focused on autoantibodies and immune complexes. T-cell-mediated antigen-specific responses (**Atsuo Nakajima et al., 2006)** and T-cell independent cytokines networks (**Firesctein G et al., 1996**) have also been implicated. The SATB1 was selected for further characterization based on the previous publications on SATB1 in which its functions were described in **(1)** densely looped, transcriptionally active chromatin for coordinated expression of cytokine genes (**Cai S et al, 2006**), **(2)** the initiation of human Th1 or Th2 cell commitment (**Lund R et al., 2005**), **(3)** nuclear targeting determinant, as a genome organizer in the T cell lineage (**Nakayama Y et al., 2005**), **(4)** the development of CD8SP thymocytes and peripheral T lymphocytes (**Nie H et al., 2005**), **(6)** tissue-specific nuclear architecture and gene expression (**Cai S et al., 2003**), **(7)** regulation of gene expression in peripheral blood T cells from patients with atopic dermatitis (**Matsumoto Y et al., 2002**), **(8)** detachment from chromatin early in T-cell apoptosis (**Galande S et al., 2001**), **(9)** in B lymphocytes quiescence, tolerance and activation of global gene expression **(Glynne R et al., 2000**), **(10)** apoptotic mechanisms (**Gotzmann J et al., 2000**) and **(11)** orchestration temporal and spatial expression of multiple genes during T-cell development (**Alvarez J.D et al., 2000**). Moreover, to our knowledge this was the first report for the presence of SATB1 in RA patient sera. EFEMP1 plays an important role in **(1)** negative regulation of chondrocyte differentiation (**Wakabayashi T et, al., 2010**), **(2)** promotion of angiogenesis and acceleration of the growth of cervical cancer in vivo (**Song E.L et al., 2011**), **(3)** unique up-regulation in malignant gliomas and promotion of tumor cell mobility and invasion (**Hu B et al., 2009**), **(4)** binding of the epidermal growth

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factor (EGF) receptor and activation of mitogen-activated protein kinase (MAPK) and serine/threonine-protein kinase (Akt/PKB) pathway in pancreatic carcinoma cells **(5)** stimulation of DNA synthesis in fibroblasts (**Lecka-Czernik B et al, 2005**), **(6)** proliferation, differentiation of synovial fibroblast cells (**Masuda K et al, 2002**) and **(7)** formation of thickness of synovial tissue layers (**Nishimura et al., 2007**).

II. Material and Methods (Pages No Enough)

III. Results

A. Transcriptomic Profiles of Clones Amplified from RA cDNA Library

Screening for novel autoantibodies was performed on cDNA phage display library, as previously described (**Somers K et al, 2009**). This screening resulted in 13 in-frame and enriched clones, of which 5 clones were derived from selections using the early RA patients, and 8 clones from selections on the RF-negative and ACCP-negative RA patients (data not shown).

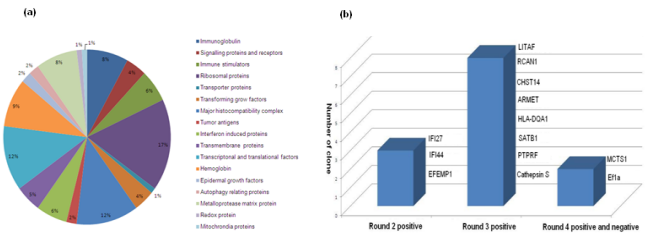

Fig. 1 Pie charts showing the detail distribution of protein profiles screened from RA cDNA library. Ribosomal proteins, immune stimulators and transcriptional and translational factor complexes that highly account for differential expressed protein is 17%, 12% and 12%, respectively.

Homology search analysis at the nucleotide and protein level showed 100% identity with DNA and amino acid sequences of known genes for these candidates on gene bank. Sequencing the cDNA insert from the clones and comparison with genome databases revealed a correspondence with peptides or proteins displayed on the phage surface. To confirm that enrichment of phage clones occurred based on specific interactions with RA patient autoantibodies and to further characterize the immunoreactivity of the identified enriched phage clones, two clones, SATB1 and EFEMP1 were expressed and purified (data not shown). The finding of SATB-1 and EFEMP-1 autoantibodies in the RA patients could be associated with overexpression of these proteins in the development of T-lymphocytes and infiltration of lymphocytes into synovial tissues.

B. Association between RA and the Presence of Circulating Autoantibodies against EFEMP1 and SATB1

To investigate the association between RA and the presence of autoantibodies directed against EFEMP1 and SATB1, immunoreactivity of 159 healthy controls and 137 RA patients against these proteins was investigated by means of ELISA.

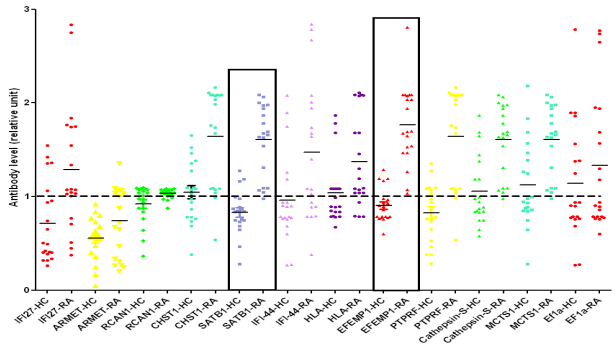

Fig. 2 Autoantibody levels were analyzed by means of protein ELISA in RA patients (n=20) and healthy controls (n=20). Background plasma reactivity was determined by measuring immunoreactivity against the V5 epitope fragment. Scatterplot dots represent the mean of OD (autoantigenic targets)/OD (V5 epitope) for each serum sample tested.

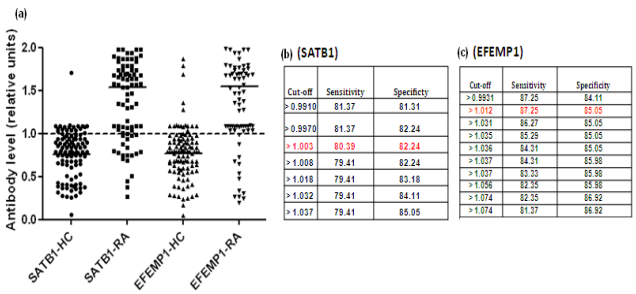

Fig. 3 Protein ELISA were performed on 137 RA patients and 159 healthy controls. Scatterplots show the level of antibody reactivity against EFEMP1 and SATB1. Background reactivity was determined by measuring the ELISA signal against the V5 epitope. The cut-off for positive samples as high as 1.5 times the standard deviation (SD) above the mean ratio OD (antigenic targets)/OD (V5 epitope) was arbitrarily set at 1 relative unit for EFEMP1 and SATB1.

Analysis of the area under the ROC curve (AUC) and p-value quantified the overall ability to test for antibody reactivity against each protein in order to differentiate between RA patients and healthy controls. The ROC results showed difference between RA patients and healthy controls by identification of autoantibody specificities

against EFEMP1 and SATB1 (Fig. 4). The presence of autoantibodies against these autoantigens via analysis of ROC AUC and p-values might contribute to diagnostic differentiation between RA patients and healthy controls. The finding provides an evidence of presence of anti-EFEMP1 and anti-SATB1 autoantibody in RA patients.

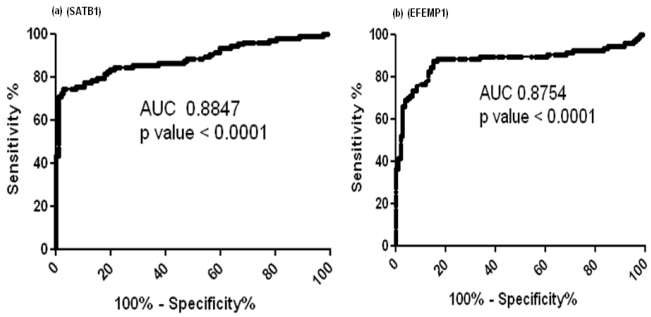

Fig. 4 ROC curves of autoantibody detection against EFEMP1 and SATB1 in RA patients and controls. The area under the ROC curve (AUC) and p-value of ROC analysis quantify the whole probability of the testing for antibodies against EFEMP1 and SATB1, to valuably distinguish between healthy controls and RA patients. The AUC value is approximately 0.5 for a useless test and 1.0 for perfectly discriminative test. The p-value of ROC was considered statistically significant when $p<0.05$.

C. *Proinflammatory Cytokines Rising Up by Stimulation of Anti-SATB1/EFEMP1 mAb*

We examined the differences in cytokine production between when cultured PBMCs after stimulation with anti-SATB1/EFEMP1 mAb.

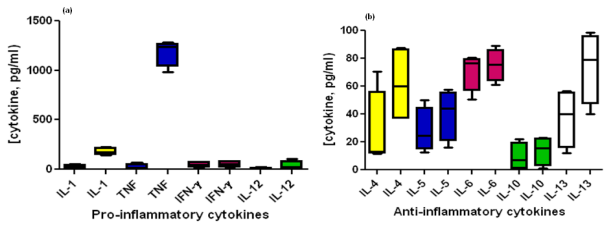

Fig. 5 Cytokine production was determined with supernatants from PBMCs culture with treatment of anti-SATB1 mAb. (a) TNF, pro-inflammatory cytokine, was stimulated up to 1000 pg/ml. (b) anti-inflammatory cytokine, significant difference from untreated cell culture assay with treated cell culture assay was measured. IL-4 and IL-13 similarly increased up to 90 pg/ml and 102 pg/ml, respectively.

Data showed a wide range of values for each of the generated cytokines. Anti-SATB1 mAb was much more effective in generating TNF synthesis than the other pro-inflammatory cytokines (**Figure 5a**). However, almost anti-inflammatory cytokines generated from treatment with anti-SATB1 mAb were variety of concentration and no significant difference from untreated and treated assay (**Figure 5b)**.

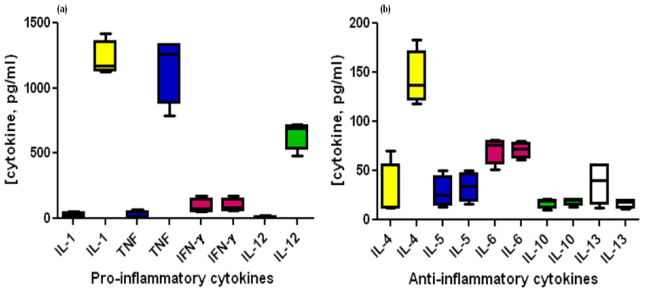

Fig. 6 Cytokine production was determined with supernatants from PBMCs culture with treatment of anti-EFEMP1 mAb. (a) TNF and IL-1, pro-inflammatory cytokine, was stimulated up to 1000 pg/ml, while IL-12 triggered up to 500 pg/ml (b) anti-inflammatory cytokine, significant difference from untreated cell culture assay with treated cell culture assay was measured. IL-4 increased up to 150 pg/ml.

The results of cytokine production following anti-EFEMP1 treatment are given in Figure 6 and again demonstrated a relatively wide range of value. In pro-inflammatory cytokine profile, TNF and IL-1 were highly synthesized **(Figure 6a)**. In anti-inflammatory cytokine profile, IL-4 production in anti-EFEMP1 mAb treated-cell culture showed wide range of values and significant difference from anti-EFEMP1 mAb treatment and un-treatment. Unexpected predominance of IL-4 secretion in PBMCs cell culture led us to hypothesize that a compensation between pro-inflammatory and anti-inflammatory cytokines.

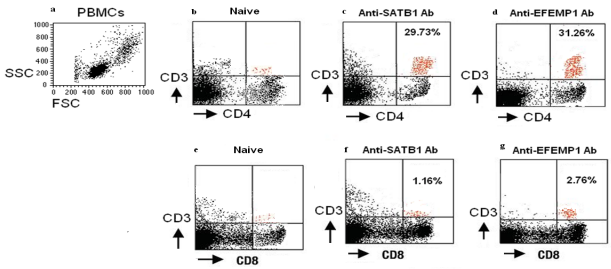

Fig. 7 Flow cytometry assay for anti-SATB1/EFEMP1 mAb-specific T cells.

(a) Lymphocytes were selected by forward and side scatter and data from at least 300,000 small lymphocytes were generally collected. (b,c,d) in most samples data from at least 30,000 to 40,000 $CD4^+$ cells were collected for analysis. (e,f,g) in most samples data from at least 30,000 to 40,000 $CD8^+$ cells were collected for analysis. Percentage of T-cell phenotypes were defined as the percentage of $CD3/CD4^+/CD8^+$ cells. A positive response was considered to be 1% $CD3/CD4^+$ or $CD3/CD8^+$ cells above background.

Examination of anti-EFEMP1 or anti-SATB mAb-stimulated $CD4^+$T in PBMCs culture showed significantly more positive results in which the percent of stimulation of 29.7% for anti-SATB1 mAb and 31.3% for anti-EFEMP1 mAb. $CD8^+$T cells from PBMCs had significantly less than $CD4^+$ that produced 1.16% for anti-SATB1 mAb and 2.76% for anti-EFEMP1 mAb. In addition to differences in the proportion of subjects, the total magnitude of cytokine-specific T cells (percentage of $CD4^+$ or $CD8^+$ T cells that had a cytokine response after anti-SATB1 or EFEMP1 mAb stimulation) was examined. In PBMCs, more $CD4^+$ highly produced TNF and IL-1 than IFN-γ and IL-12.There is no difference in the percentage of $CD8^+$T cell followed by treatment of anti-SATB1/EFEMP1 mAb (Figure 7f,g).

IV. Discussion

In the light of autoantibody profiles in analysis of the humoral immune response in RA, finding of biomarkers for early and RF-negative and ACCP-negative RA and analysis of the autoantibody profiles in these patients can supply important insights into RA pathogenesis. The enriched and in-frame phage clones presented in the RA phage display library reflected the presence of the identical autoantibodies in serum, which suggests an essential involvement of these antibodies in the aetiology and disease process of RA. Moreover, these findings correlate with mechanisms of long-lived plasma and memory B cells, including autoreactive cells (**Dorner T et al, 2009**). Elevated immunoreactivity could only be found in the RA group (137 patients) compared to the control group (159 patients) (Fig. 3). Moreover, differences in immunoreactivity did allow discriminating between healthy controls and RA patients. EFEMP1 in early RA may be associated with proliferation and differentiation of synovial fibroblast cells (**Masuda K et al, 2002**). Interestingly, EFEMP1 stimulates DNA synthesis in fibroblasts (**Lecka-Czernik B et al, 2005**), whereas SATB1 is a genome organizer connecting multiple genomic loci and recruiting specific enzymes for chromatin-remodelling and gene expression (**Han H.J et al, 2008**). Therefore, the presence of autoantibodies against EFEMP1 and SATB1 in RA patients might be interrelated. In 137 RA patients, the discrimination of antibody levels against EFEMP1 and SATB1 were significant. Power of test on screening of the sample group including 137 RA patients and 159 healthy controls showed sensitivities (87%) and specificities (52%) for the EFEMP1 markers for early RA and RF(-) anti CCP (-) RA. Clinical parameters of fibrosis score and expression level associating with EFEMP1 expression (**Masuda K et al, 2002**) indicated that EFEMP1 could be involve in the pathology of ACCP-negative RA patients through regulation of MMPs.

SATB1 essentially regulates chromatin-loop architecture and transcription and organization of the major histocompatibility (MHC) class I locus into higher-order chromatin-loop structures (**Pavan K P, et al., 2007**). Silencing SATB1 by small interfering RNA (siRNA) or mutating SATB1 alters chromatin architecture and affects the expression profile of cytokine genes. Although the role of B cell subsets in RA is unclear, anti-SATB1 antibody found in serum of RF-negative and ACCP-negative RA patients might play a role in differentiation of pre-mature B cells into RF-negative and ACCP-negative RA associated B cell subsets.

Another study showed that SATB1 promotes lymphocyte regulation and production from primitive hematopoietic stem and progenitor cells (**Wen J.H et al., 2004**). A suggestion is whether the absence of RF and ACCP-antibody in the RA sub-population is regulated at the level of hematopoietic stem cells and progenitor cells. High-affinity antibodies could be relevant to pathogenesis and early RA diagnostics. Nonetheless, no association existed between antibody positivity towards these antigenic targets and available clinical parameters. A larger population testing with different disease subtypes could be possible to find antibody positivity with clinical parameters. Another hypothesis is that under SATB1 regulation at genome level in lymphocytes, a different B-cell subpopulation is differentiated from stem lymphocytes.

This study determined normal ranges for IL-1, IFN-γ, IL-12 and for IL-5, IL-6, IL-10 followed by anti-SATB1 mAb stimulation. Anti-EFEMP1 mAb showed normal ranges for IFN-γ and for IL-5, IL-6, IL-10. Increased levels of IL-1 and TNF noted to anti-SATB1/EFEMP1 mAb might have been due to dual stimulation of both T and B lymphocytes. The B cells might have produced an amplificative factor which then stimulated the T lymphocytes to increase IL-1, TNF production. The association between cytokine levels for each autoantibody and for all individuals was assessed. Stimulation with these autoantibody demonstrated direct correlations between pro-inflammatory could be stimulated and regulated by anti-SATB1/EFEMP1 autoantibody. Interleukin-1 (IL-1) and tumour necrosis factor (TNF) produce inflammatory and tissue destruction (**Charles A et al., 2000**). This indicates that anti-EFEMP1 mAb could play both functions of direct tissue destruction and IL-1 and TNF-mediated tissue destructinon. IL-4 is potent anti-inflammatory agents; it suppresses genes for pro-inflammatory cytokines such as IL-1 and TNF. This suggests that the larger magnitude reflected responses from population of T cells that regconized a large diversity of cytokine gene products rather than a single population of T cell that regconized a single gene product. The mechanism of how the autoantibodies generated the cytokine production was not known. IL-5 is involved with

eosinophil proliferation. Therefore, in the healths would not expect the lymphocyte to be primed to produce increased IL-5 levels. Cytokine levels vary not only in cell differentiation in vitro but also in vivo of different clinical disease. The roles of cytokines within a complex regulatory network are related to specific immunological, chronic inflammation and tissue destruction. In synovial lavage of individuals with very early rheumatoid arthritis revealed elevated levels of IL-4 and IL-13 (**Raza et al., 2005**) and can stimulate T cell proliferation. In addition, IL-1 elicits of histamine from mast cell.TNF and IL-1 share several pro-inflammatory properties. Both cytokine can stimulate the production of collagenase by synovial cells (**Warren J.S et al., 1990**).

How autoantibodies such as anti-SATB1 mAb and anti-EFEMP1 mAb, known to stimulate the T-cell differentiation, could first stimulate IL-1, TNF and IL-4 synthesis and then involved into T cell differentiation. To elucidate the roles of SATB1 and EFEMP1 antibodies in RA, cytokine assay and T-cell phenotyping, functions of $CD4^+$ T cells are mediated by cytokines. The increasing of the IL-1, TNF and IL-4 in cytokine assays was associated with rising up of $CD4^+$T cell percentage. Anti-SATB1 mAb stimulation also demonstrated a positive relation of TNF and IL-13, and IL-4, and fit the elucidation of $CD4^+$/$CD8^+$ T cells (**Borish L et al., 1996).** The explain of why the each autoantibody stimulated the cells to produce different levels of cytokines was not clear, although the FACS determined the formation of the stimulated T cell subsets from PBMCs culture.

It is through that antibodies specific for nuclear autoantigens are frequently present in serum of RA patients, however their specificity and sensitity are often low and are used as an additional test when RF and anti-CCP antibodies showed an unclear results (**Lorenz H.M et al., 2005**). Understanding of their functions in RA will direct further research on the antigens that are associated with these autoantibodies in RA. It is useful for development of novel diagnostic approaches to identify the expression profiles of antigenic targets in RA associated cells, should be further investigated for their use as novel biomarkers or additional biomarkers in available validated biomarker panels.

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The below research sections were performed at Biomedical Research Institute, Transnational University Limburg, Belgium.

1. Amplification, fingerprinting and sequencing of the clones retrieved from cDNA phage display library
2. Cloning, expression and immunoblotting of SATB1 and EFEMP1
3. Mass spectrometry (MS)

Flow cytometry were researched at GBEC Bioengineering Company limited and Protein ELISA was done at Biotechnology Center of Ho Chi Minh City.

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