Published in final edited form as:

Genes Immun. 2009 June ; 10(4): 285–296. doi:10.1038/gene.2009.20.

Peripheral blood gene expression profiling in Sjögren's syndrome

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Abstract

Sjögren's syndrome (SS) is a common chronic autoimmune disease characterized by lymphocytic infiltration of exocrine glands. Affected cases commonly present with oral and ocular dryness, thought to be the result of inflammatory cell-mediated gland dysfunction. To identify important molecular pathways involved in SS, we used high-density microarrays to define global gene expression profiles in peripheral blood. We first analyzed 21 SS cases and 23 controls and identified a prominent pattern of overexpressed genes that are inducible by interferons (IFNs). These results were confirmed by evaluation of a second independent dataset of 17 SS cases and 22 controls. Additional inflammatory and immune-related pathways with altered expression patterns in SS cases included B and T cell receptor, IGF-1, GM-CSF, PPARα/RXRα, and PI3/AKT signaling. Exploration of these data for relationships to clinical features of disease revealed that expression levels for most IFN-inducible genes were positively correlated with titers of anti-Ro/SSA ($P<0.001$) and anti-La/SSB ($P<0.001$) autoantibodies. Diagnostic and therapeutic approaches targeting IFN signaling pathway may prove most effective in the subset of SS cases who produce anti-Ro/SSA and anti-La/SSB autoantibodies. Our results strongly support innate and adaptive immune processes in the pathogenesis of SS and provide numerous candidate disease markers for further study.

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Introduction

Lymphocytic infiltration into exocrine glands is a hallmark of Sjögren's syndrome (SS) pathogenesis. Disruption of target organ function, particularly salivary and lacrimal gland secretion, may lead to severe and irreversible damage. The extent to which the exocrinopathy affects saliva and tear production varies, but moisture can be virtually nonexistent and lead to corneal scarring, blurred vision, rampant dental caries, recurrent oral infections, and difficulty with speaking, swallowing and eating[1; 2]. Extraglandular manifestations in SS are also common, heterogeneous, and may involve the skin and genitourinary tracts, as well as the hematologic, neurologic, respiratory, gastrointestinal, vascular, and musculoskeletal systems. Approximately half of SS cases experience lymphocytic mediated organ damage[3]. Increased risk of lymphoma in SS cases has been established, with estimates as high as 44-fold[4]. Approximately half of SS cases present an accompanying autoimmune rheumatic disease, most commonly rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or scleroderma[5].

The molecular basis of SS is not well defined, but includes production of autoantibodies, dysfunction of molecular water transport processes, dysregulation of apoptosis, and cytokine activity abnormalities[4; 6–8]. A role for viral infection in SS has long been suspected but difficult to establish. Numerous viruses have been considered, including Epstein-Barr virus (EBV), cytomegalovirus, hepatitis C virus, human herpes virus 6, coxsackie virus and several retroviruses[9; 10]. Specific evidence supporting these candidate viruses vary, but include such properties as the ability to directly infect cells in the salivary gland and/or immune system, sequence similarities between viral proteins and autoantigens (particularly La/SSB) suggesting molecular mimicry, elevation of viral antibodies or viral sequences, association between viral infection and lymphoma, and association of symptoms mimicking SS following viral infection. Regardless of the specific virus, mechanisms of host-virus relationships that control or perpetuate latency/re-activation cycles of viral replication and inflammatory responses, such as production of IFNs, are likely to be important in SS.

Multiple genes are thought to increase disease susceptibility to SS, including human leukocyte antigen (HLA) loci, interleukin 10 (IL-10), Fas, Fas ligand (FasL), and more recently, interferon regulatory factor 5 (IRF5)[11; 12]. Other polymorphisms have been found to be associated with various clinical features of SS. For example, association of anti-Ro/SSA autoantibody with the 52kD Ro/SSA gene[13], Ig KM and GM genes with clinical presentation of SS[14], and apoE with early onset of SS have been described[15].

Developments in high-throughput transcriptional profiling employing microarray technology have dramatically expanded our ability to identify key molecular pathways related to disease. Previous studies using microarray approaches in SS have been limited to studies of salivary tissue in relatively small cohorts of cases. These studies have identified over-expression of interferon (IFN)-inducible genes in salivary gland tissue from SS cases[16; 17], similar to that seen in other autoimmune diseases[18].

The identification of biomarkers for SS in peripheral blood mononuclear cells (PBMCs) or whole blood (WB) cells offers a very practical alternative to current approaches for

diagnosis and classification of SS cases[19]. Furthermore, peripheral blood has proven to be informative for advancing our understanding of related autoimmune diseases including SLE, RA, psoriasis, and multiple sclerosis[18]. In the present study, we sought to identify important disease associated pathways and explore correlations of gene expression profiles to relevant clinical features of SS.

Results

Identification of an IFN-inducible gene signature in peripheral blood mononuclear cells of SS cases

As an initial discovery effort, global mRNA transcript levels were measured in PBMCs of 21 SS cases and 23 healthy controls (Cohort 1) using Affymetrix U95A2 GeneChips containing 12,626 oligonucleotide probe sets. Demographic features of participant cohorts are shown in Table 1. To identify differentially expressed transcripts between SS cases and controls, we used three data filtering criteria: Welch *t*-test *P*-value ≤ 0.001, mean fold change ≥ 1.5 and mean expression difference ≥ 100. A total of 425 mRNA transcripts representing 382 unique genes were identified as differentially expressed in SS cases (Figure 1A, Supplementary Table 1). Approximately 40 genes were identified more than once by multiple probesets on the Affymetrix arrays. Significance levels for some transcripts reached *P*-values < 10^{-14} and fold-changes as high as 24 (Table 3, Supplementary Table 1). We observed 129 overexpressed and 296 underexpressed mRNA transcripts in SS cases relative to controls.

Unsupervised hierarchical cluster analysis was conducted to visualize patterns of the 425 differentially expressed transcripts (Figure 1A). Of the 129 overexpressed transcripts, 46% (n=59) are known to be inducible by IFNs (Figure 1A, Supplementary Table 1). Genes in this cluster include interferon-induced protein 35 (IFI35, $P = 1.34 \times 10^{-11}$), myxovirus (influenza virus) resistance 1 (MX1, $P = 9.94 \times 10^{-8}$), 2′5′-oligoadenylate synthetase 1 (OAS1, $P = 1.05 \times 10^{-7}$), interferon regulatory factor 7 (IRF7, $P = 1.98 \times 10^{-7}$), and OAS 2 ($P = 3.15 \times 10^{-7}$).

We then used INGENUITY PATHWAYS ANALYSIS (IPA) software (ver. 5.5) to facilitate the systematic identification and grouping of differentially expressed genes into biological networks. Fifty-nine functional categories were identified by IPA as statistically significant for the 425 differentially expressed transcripts. Table 2 presents the top 20 most significant biological function categories (see Supplementary Table 2 for a list of all functional categories and sub-categories). Cell death was the most significant biological function with sub-category p-values ranging from 2.55×10^{-11} to 2.96×10^{-3}, followed by cellular growth and proliferation ($P = 3.67\times10^{-8}$ to 1.72×10^{-3}) and immune and lymphatic system development and function ($P = 2.39\times10^{-9}$ to 2.83×10^{-3}).

IPA also identified 42 statistically significant canonical pathways from our list of differentially expressed transcripts in Cohort 1 (Supplementary Table 1). As shown in Figure 2, IFN signaling was the most significant pathway ($P = 1.57\times10^{-5}$) followed by B cell receptor signaling, IGF-1 (insulin-like growth factor-1) signaling, GM-CSF (granulocyte macrophage-colony stimulating factor) signaling, PPAR (peroxisome

proliferator-activated receptor) signaling, PPARα/RXRα activation, T cell receptor signaling, PI3/AKT (phophatidylinosital 3-kinase) signaling, acute phase response signaling, and JAK/STAT (janus kinase/signal transducer and activator) signaling among others (Figure 2). In general, transcripts involved in IFN signaling and protein ubiquitination were largely overexpressed while the majority of transcripts from other pathways identified were underexpressed in SS cases versus controls. Significant overlap of differentially expressed genes was apparent across the 42 canonical pathways. For example, five genes (RRAS, KRAS, PIK3CA, PIK3R1, PIK3CG) are multifunctional transcription factors or signaling molecules involved in over 20 of the 42 canonical pathways we identified. In addition, over 57% of the genes shown in Figure 2 mapped to the top 9 most statistically significant pathways ($P < 0.001$) identified by IPA. Within these 9, two sets of pathways were closely related: PPARa/RXRa activation/signaling and B cell/T cell receptor pathways. Of the remaining 33 pathways, 15 consisted entirely of genes that directly overlap with other pathways in Figure 2.

Replication of the IFN-inducible gene signature in whole blood of SS cases

We next evaluated an independent group of 17 cases and 22 controls (Cohort 2, Table 1). Affymetrix U133A GeneChips with an expanded representation of 22,283 oligonucleotide probe sets were used to measure RNA transcript levels in this independent Cohort. In addition to expanding the overall number of transcripts assayed in Cohort 2, we were also able to utilize more recently developed blood collection procedures that stabilize RNA transcript levels at the time of phlebotomy (see Methods). As opposed to selecting a few transcripts for validation studies of our results from Cohort 1 (commonly done by quantitative PCR), this comparison provided a much more comprehensive approach for confirmation of the differentially expressed pathways through replication in an independent set of cases and controls.

Using the same 3-step data filtering approach (Welch *t*-test *P*-value ≤ 0.001, mean fold change ≥ 1.5 and mean expression difference ≥ 100), 120 RNA transcripts in 100 genes (18 underexpressed and 102 overexpressed) were identified as differentially expressed in cases relative to controls (Supplementary Table 3). Cluster and pathway analysis of significant transcripts were used to identify gene expression patterns (Figure 1B, Figure 2). Similar to the results in Cohort 1, the prominent signature of overexpressed IFN-inducible genes was observed in Cohort 2 (Figure 1B). Comparison of differentially expressed transcript lists for Cohort 1 and Cohort 2 resulted in identification of a total of 38 genes common to both cohorts, the majority (n=34, 89%) of which represented IFN-inducible transcripts (Table 3). Thus, these genes represent a reproducible “IFN signature” identifiable in peripheral blood of SS cases.

Table 4 provides the results for selected IFN and IFN pathway regulators in both Cohorts 1 and 2. In general, the majority of IFN genes encoding the IFNs themselves were not differentially expressed in peripheral blood. In contrast, interferon regulatory factor 7 (IRF7), a key transcription factor involved in downstream signaling events triggered through IFN or Toll-like receptors, was upregulated by over 2-fold in both datasets (Cohort 1

P=5.57×10^{-6}, Cohort 2 P=1.98×10^{-7}). Approximately 66% of the transcripts in this group were differentially expressed by 2-fold or greater in at least one cohort.

It is possible that the difference in age observed between cases and controls in Cohort 1 (mean for cases = 57, mean for controls = 31) may have contributed to a larger number of differentially expressed transcripts indentified in Cohort 1 (n = 425 in Cohort 1 and n = 120 in Cohort 2). However, we believe that the use of whole blood in Cohort 2 is likely to have a greater impact in our ability to detect differential expression, since an excess of globin transcripts in whole blood microarray experiments has been shown to mask signatures of biological relevance and produce fewer significant results when compared directly with PBMCs. Moreover, a direct comparison of our list of differentially expressed genes observed in Cohort 1 with a list of genes related to aging provided by the GenAge Database (http://genomics.senescence.info/genes/) resulted in very few overlaps (n=15). Additional evidence to support association with SS for all of these 15 genes exists, either through results of other undergoing microarray studies (Moser KL, unpublished data), inclusion in significant biological pathways with several other genes identified as differentially expressed in this study, or previous reports in the literature. Finally, despite the difference in number of differentially expressed transcripts between Cohorts 1 and 2, many of the significant canonical pathways were observed in both Cohorts. Using IPA, 10 statistically significant canonical pathways were identified in Cohort 2, nine of which were also observed in Cohort 1 (Figure 2, indicated in bold). One additional pathway, antigen presentation, was statistically significant in Cohort 2 (P=0.0025). In Cohort 1, the antigen presentation pathway was ranked 43rd and fell just below the threshold for significance (P=0.056).

Thus, using two independent cohorts, alternative versions of microarray GeneChips, and varying sample compositions of either PBMCs (Cohort 1) or WB (Cohort 2), we have observed consistent, reproducible overexpression of IFN-inducible gene expression patterns and identified several additional pathways characterized by downregulated patterns of gene expression in pSS cases compared with normal controls.

Correlation of IFN-induced gene expression patterns and key clinical features

We next wanted to explore the association between dysregulated pathways and clinical measures of SS. To maximize our statistical power, we generated a third, larger dataset consisting of a total of 36 SS cases and 22 controls (Cohort 3). All data for Cohort 3 was generated from whole blood using the Affymetrix U133A GeneChips containing 22,283 probesets. We combined all available data from Cohort 2 with new data from Cohort 1 subjects who were resampled using PAXgene tubes (and thus, assayed from whole blood and assayed using the U133A GeneChip to be amenable for combining with Cohort 1 data). Because Cohort 3 included all subjects from both Cohort 2 and most subjects from Cohort 1, analysis of this third dataset was not considered independent from the results described above, but did allow more statistically robust results given the large sample size for correlation analyses (see Methods). The clinical variables evaluated included saliva production measured by whole unstimulated salivary flow (WUSF), tear flow measured by

Schirmer's test (ST), and titers of anti-Ro/SSA and anti-La/SSB autoantibodies determined by ELISA.

Figure 3a shows the hierarchical cluster graph of 223 RNA transcripts in 193 genes (197 overexpressed and 86 underexpressed) identified as differentially expressed between Cohort 3 SS cases and controls using the three-filter criteria (Supplementary Table 4). The distributions of clinical variable values are shown in Figure 3b. Correlation coefficients for RNA expression values with tear flow, salivary flow and autoantibody titers (measured in the same sample for each individual) were estimated for the 223 differentially expressed RNA transcripts in the group of 36 SS cases. Healthy controls were not included in these analyses so that we could assess significant correlations defined within the case group only.

As shown in Figure 3c, most of the correlation tests did not reach statistical significance ($P > 0.05$) for salivary flow or tear flow (WUSF and ST, respectively). This is an expected result since all SS cases are ascertained based on reduced values for these clinical variables. Of the 223 RNA transcripts, only 11 were significantly correlated with salivary flow (5%) and 17 for tear flow (8%). Of the 86 underexpressed RNA transcripts, ≤ 6% correlated with titers of anti-Ro/SSA and anti-La/SSB autoantibodies (3 and 5 transcripts, respectively). In contrast, a large proportion of the 197 overexpressed RNA transcripts were positively correlated ($P < 0.05$) with titers of anti-Ro/SSA (n=89 or 45% of the transcripts) and anti-La/SSB (n=76 or 39% of the transcripts). Approximately two-thirds of the RNA transcripts that were correlated with anti-Ro/SSA and/or anti-La/SSB autoantibodies are known to be IFN-inducible genes. Correlations between the clinical variables tested and transcripts involved in other dysregulated pathways identified in Cohorts 1 and 2 (e.g. B/T cell receptor signaling, IGF1R, GM-CSF signaling, etc.) were not observed (Figure 3).

Discussion

We have applied microarray technology to define global gene expression profiles in pSS and identified several key pathways that are dysregulated in cases versus normal controls. Our study is the first to demonstrate that upregulation of IFN-inducible gene expression is prominent in peripheral blood cells of many SS cases, and correlates with high titers of anti-Ro/SSA and anti-La/SSB. In addition, analysis of two independent cohorts revealed evidence for dysregulation of signaling through the B cell/T cell receptors, IGF-1, GM-CSF, PPARα/RXRα, and several cytokine pathways that appear to be consistent across all SS cases.

Microarray-based studies in human pSS have previously focused on the identification of disease associated pathways in saliva or in minor salivary gland tissue from relatively small cohorts (10 or fewer cases plus controls)[16; 17; 20; 21]. A common finding across the four studies reported to date is upregulation of IFN-inducible genes. Genes overexpressed in our data generated using peripheral blood that have also been reported as upregulated in minor salivary glands and/or saliva from SS cases include interferon-induced transmembrane proteins 1 (9–27, IFITM1) and 3 (1-8U, IFITM3), promyelocytic leukaemia (PML), transporter 2 ATP-binding cassette (TAP2), spleen tyrosine kinase (SYK), guanylate binding protein, 2 (GBP2), and interferon-induced protein 44 (IFI44)[16; 17; 20]. These genes

and others that show similar consistency across multiple sample types underscore both the local and systemic nature of IFN pathway dysregulation. Furthermore, these genes may serve as especially attractive targets for development of clinically useful biomarkers. Disease markers that are both central to pathology in target tissues (e.g. salivary glands) and potentially more feasible to assay through saliva or serum-based diagnostic tests would provide a significant improvement over the current approaches to classification of SS cases.

In recent years, upregulation of IFN pathway signaling has been noted in a growing list of autoimmune disorders, including psoriasis, multiple sclerosis, rheumatoid arthritis, dermatomyositis, primary biliary cirrhosis, and insulin-dependent diabetes mellitus [18]. The IFN-inducible gene expression profile we report in SS is remarkably similar to the "IFN signature" that has been observed in similar studies of peripheral blood in SLE, present in a majority of cases[22] (Moser KL, unpublished observations). In addition to overlap of certain clinical features in both SLE and SS, production of anti-Ro/SSA and anti-La/SSB autoantibodies are common in both disorders. In our study, the IFN signature in SS was significantly correlated with high titers of anti-Ro/SSA and anti-La/SSB. Although the precise underlying disease mechanism connecting IFN pathway activation and autoantibody production is unclear, these results provide further support to link both innate and adaptive immune responses to the pathogenesis of disease.

Activation and control of IFN-inducible genes may be dysregulated due to abnormal levels or activity of a class of transcription factors known as interferon regulatory factors (IRFs). For example, IRF-1 and IRF-2 are structurally similar DNA-binding factors which were originally identified as regulators of the type I IFN system; IRF-1 functions as a transcriptional activator, and IRF-2 represses IRF-1 function by competing for the same cis elements[23]. Evidence from our data sets suggests IRF-1 is upregulated and IRF-2 is downregulated in SS cases. Such an imbalance is consistent with upregulation of IFN-inducible genes. Furthermore, IRF-5 and IRF-7, both upregulated in our data, play a crucial role in the expression of type I IFN genes, cytokines and some chemokines[24; 25]. Interestingly, EBV regulates and uses IRF-7 as a secondary mediator for several target genes involved in latency and immune regulation. In addition, Ning *et al.* have demonstrated that the virus activated factor of Sendie virus binds to IRF7 IFN stimulating element and can directly activate IRF7 transcription independent of IFN-triggered JAK-STAT pathway [26]. Finally, genetic association of polymorphisms in IRF5 and STAT4, directly involved in IFN pathway signaling, with both SLE and SS has been reported[12; 27–30]

Collectively, these observations indicate that overexpression of IFN responding genes in SS may result not from overexpression of IFN genes themselves but rather from effects mediated more directly by viral infection and/or genetic variants in IRFs and other IFN pathway mediators that contribute to altered signaling. The potential role of the Type I interferon system in SS was recently reviewed by Nordmark *et al*[31]. Current data supports a mechanism of disease in which an initial viral infection induces Type I interferon production in salivary glands, leading to apoptosis or necrosis of glandular epithelial cells and exposure of autoantigens such as anti-Ro/SSA and anti-La/SSB followed by activation of adaptive immune responses (both locally and systemically). Production of autoantibodies (including anti-Ro/SSA and anti-La/SSB) that form immune complexes with nucleic acids may trigger

prolonged activation of IFN pathways through Toll-like receptor-medicated stimulation of plamacytoid dendritic cells[31]. Additional production of IFNs, as well as cytokines known to be relevant to SS including, IL-12, IL-6, TNF, CXCL10, and CCL3, can be produced by pDCs, leading to recruitment and perpetuation of a continuous cycle if not properly downregulated[32]. Consequently, this process leads to impaired function of affected exocrine glands and potential systemic manifestations commonly seen in SS patients. Our results showing correlations between IFN pathway activation and autoantibodies bring up important considerations for the development of improved diagnostic and therapeutic strategies. We propose that development of biomarkers which reflect the IFN signature and therapies directed against IFN pathway activation are most likely to be successful in the subset of patients with high-titers of anti-Ro/SSA and/or anti-La/SSB.

IPA identified 59 functional categories associated with the list of differentially expressed genes identified in Cohort 1. We found these categories to be too broad for the development of hypotheses of disease mechanisms, and as a result, have focused our attention on canonical pathways. In addition to upregulation of an IFN-inducible gene expression pattern, we identified over 40 additional canonical pathways that were differentially expressed in our PBMC dataset using IPA. However, these pathways do not appear to be independent of each other. Close examination of the genes included in these pathways revealed a significant amount of overlap, most likely reflecting the extensive “crosstalk” that occurs among closely related biological pathways. These results suggest that certain pathways, such as those initiated through B or T cell receptor signaling, account for the seemingly large number of the pathways identified by using approaches such as IPA.

Several of the canonical pathways and dysregulated genes (outside of the “IFN signature”) represent interesting and potentially important new avenues for further investigation. For example, B cell/T cell receptor signaling was significantly dysregulated in this study. One of the genes in these pathways, PTPRC or protein-tyrosine phosphatase, receptor-type, C (also known as CD45, CD45R, and Ly5), is a major leukocyte cell surface molecule that suppresses JAK kinase and negatively regulates cytokine receptor signaling [33]. PTPRC is essential for activation of T cells and B cells, and important for integrin-mediated adhesion and migration of immune cells. In our data, PTPRC was overexpressed in cases versus controls, consistent with enhanced downregulation of other B/T cell pathway genes observed. Targeted disruption of PTPRC has been shown to enhance cytokine and interferon receptor-mediated activation of JAK and STAT proteins[33]. Furthermore, genetic associations of variants in PTPRC have been reported with multiple sclerosis, Grave’s disease and Hashimoto’s thyroiditis[34]. In murine models, genetic variants in PTPRC lead to lymphoproliferation and severe autoimmune nephritis with autoantibody production and alterations in cytokine production. Thus, evaluation of PTPRC and other related genes in lymphocyte signaling pathways may be informative in further defining autoimmune responses in SS.

The insulin-like growth factor 1 receptor (IGF1R) was underexpressed in our study, consistent with a study of SS minor salivary glands by Katz *et al*[35]. Low levels of IGF1R have also been shown in the non-obese diabetic mouse model of experimental autoimmune sialadenitis[36]. Dysregulation of this pathway may result in the inability of IGF-1 to exert its

homeostatic, protective effect in salivary tissue and lead to glandular atrophy and disfunction[35].

Altered signaling through PPARα/RXRα pathways also offers intriguing clues to SS pathogenesis. PPARs (peroxisome proliferator-activated receptors) are nuclear receptors that when activated by ligand, form a functional transcriptional unit upon heterodimerization with retinoid X receptors (RXRs) [37]. PPARα and related family members are critical modulators of environmental and dietary stimuli, and play a key role in downregulating inflammatory responses[37; 38]. In immune cells, PPARα inhibits inflammatory pathways through sequestration and repression of c-jun and NF-κB transcription factors[38; 39]. Underexpression of PPARα in SS cases relative to controls, as observed in our study, is thus consistent with a pro-inflammatory process. Interestingly, studies in experimental autoimmune encephalitis, a murine model of multiple sclerosis (MS), have demonstrated baseline lower expression levels of PPARα in CD4+ T cells from females relative to males, resulting in increased NF-κB and c-jun activity, higher production of IFN γ and tumor necrosis factor and thus, differential regulation of PPARα between genders may contribute to increase risk of disease in females with MS and other autoimmune diseases[40]. Agonists of PPARα have been proposed as a potential therapeutic approach in MS and several other autoimmune and inflammatory disorders associated with decreased PPARα expression such as psoriasis and atopic dermatitis[41]. Furthermore, PPARα agonists have been proposed as an effective therapeutic intervention for treatment of dry eye in SS[42]. Thus, further studies should be considered to explore the potential application of PPARα agonists as novel therapeutic agents.

In summary, using varying peripheral blood cell populations (mononuclear cells and whole blood), two independently collected cohorts of cases and controls, and two different versions of Affymetrix GeneChips (U95A and U133A), we have shown a consistent upregulation of IFN inducible genes in SS cases. Our results further show that this pattern is most prominent in the subset of cases serologically defined by increased titers of anti-Ro/SSA and anti-La/SSB autoantibodies. We also identified numerous additional signaling pathways that collectively support a significant role for both innate and adaptive immune dysregulation in SS. These results should foster multiple lines of further investigation including genetic and functional studies that will hopefully lead to new insights into pathogenesis of this complex autoimmune disorder.

Methods

Case characteristics

All protocols used in this study were approved by the University of Minnesota Institutional Review Board. All participants provided written informed consent before entering the study. All SS cases met the 2002 Revised European Criteria proposed by the American European Consensus Group (AECG)[19]. Accordingly, cases were classified with SS if they had an autoimmune component (detection of anti-Ro/SSA and/or anti La/SSB autoantibodies) and/or evidence of lymphocytic infiltration through labial salivary gland histopathology, plus characteristic symptoms (dry eyes and dry mouth) and signs (decreased tear flow measured by Schirmer's test or decreased unstimulated whole salivary flow). Cohorts 1 and

2 consisted of independent subjects. Cohort 3 included 19/21 cases from Cohort 1 (samples redrawn, see below) plus all 17 cases from Cohort 2. Two of the cases in Cohort 1 also met the ACR criteria for SLE.

Controls were asymptomatic for dry eyes, dry mouth, and had no self-reported family history of autoimmune diseases. The first group of controls (n=17) consisted of all female Caucasians with an average age of 31, which were used for comparison with Case Cohort 1. The second group of controls (Cohort 2) was all female with 21/22 reporting Caucasian ancestry. These controls had a mean age of 51 and were used for analysis of both Case Cohorts 2 and 3.

Data collection procedures consisted of subject interviews, completion of a detailed questionnaire, review of medical records, physical examination, Schirmer's test without anesthesia (5 minutes), unstimulated salivary flow measurement (15 minutes), and phlebotomy for RNA extraction and determination of anti-Ro/SSA and anti-La/SSB autoantibodies.

Sample Preparation and Hybridization

Total RNA was extracted from PBMCs by Trizol (GIBCO/BRL, Invitrogen, Carlsbad, CA) or from whole blood using the PAXgene Blood RNA method (QIAGEN/BD, Valencia, CA). The methods for preparation of complimentary RNA (cRNA) were provided by the manufacturer (Affymetrix, Santa Clara, CA; GeneChip technical manual). Briefly, 5 to 10 mg of total RNA of each sample was converted into double stranded complimentary DNA (cDNA) using a Superscript cDNA synthesis kit (Invitrogen, Carlsbad, CA) with a oligo(dT)24 primer. After second-strand synthesis, labeled cRNA was generated from the cDNA sample by an in vitro transcription (IVT) reaction using BioArray labeled biotin ribonucleotides (Enzo, New York, NY). The labeled cRNA was purified using RNAeasy spin columns (Qiagen, Valencia, CA). Fifteen micrograms of each cRNA sample was fragmented by mild alkaline treatment, at 94°C for 35 min in fragmentation buffer (Tris Acetate PH.8.1/1M, 150 mM MgoAc and 500mM KoAc). Fragmented cRNAs were hybridized to Affymetrix Human U95Av2 or U133A GeneChips.

All Cohort 1 samples were collected in CPT tubes and processed within 4 hours of phlebotomy. However, given *ex vivo* changes that can be observed in expression levels for a substantial fraction of genes shortly after phlebotomy[43], whole blood was directly collected into PAXgene tubes for Cohorts 2 and 3, which contain an RNA stabilizing agent. As a result, blood sample composition for Cohorts 2 and 3 (whole blood) were different than for Cohort 1 (peripheral blood mononuclear cells). A total of 19 subjects were drawn twice; first for inclusion in Cohort 1 and later for inclusion in Cohort 3.

Anti-Ro/SSA and anti-La/SSB autoantibody assays

The levels of anti-Ro/SSA and anti-La/SSB autoantibodies in the serum of SS cases and controls were measured by ELISA (Immunovision, Springdale, AR). Absorbance was measured at 490 nm. The cutoff absorbance value above which antibody levels were

considered positive was set to the mean plus 2-times the standard deviation of titer values for controls.

Data Processing

Initial data processing involved several quality control checks assessing the starting and amplified RNA and the overall hybridization process. Quality control criteria included: 1) the ratio of 3′ to 5′ probe sets should be less than 3; 2) more than 30 percent of genes should be called 'present'; and 3) the murine sequences received an 'absent' call while human "housekeeping" sequences received a 'present' call.

We used GeneData Expressionist database and software (http://www.genedata.com) for further processing and analyzing the data. The MAS 5.0 (Affymetrix Microarray Suite 5) algorithm was used for data normalization. Gene expression intensity for each array was scaled to 1500 intensity units to allow comparison across all arrays. The scaled expression intensities were imported into GeneData Analyst (version 4.2) for statistical analysis.

Gene selection for hierarchical cluster analysis

In all 3 Cohorts, transcripts were defined as differentially expressed and selected for cluster analysis if, for the mean comparison between SS cases and healthy controls, the following criteria were met: 1) *P*-value of 0.001 or less from Welch *t*-tests; 2) change in mean expression of at least 1.5-fold; 3) mean expression difference of at least 100 units[22]. Hierarchical cluster analysis was applied to the 3 datasets using CLUSTER software and visualized using TREEVIEW software[44].

Correlation of gene expression and clinical variables

Pearson correlation estimates and p-values between transcript levels and clinical variable measurements (anti-Ro/SSA, anti-La/SSB, WUSF, and ST) were computed for each of the differentially expressed transcripts. *P*-values of correlations for each transcript were plotted as a moving window average across units of 5 transcripts[45].

Identification of canonical pathways

INGENUITY PATHWAYS ANALYSIS (IPA; version 5.5) software (https://analysis.ingenuity.com) was used to determine significant functional categories and canonical pathways based on our lists of significant transcripts. IPA tests associations between specified genes and sets of functional genes that are part of biologically relevant networks according to literature findings. Right-tailed Fisher's exact tests are used to measure the likelihood that such associations are due to chance. The proportion of genes mapped to a specific canonical pathway that are specified by the user is taken into account for the computation of *P*-values.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by NIH NIAMS RO1 AR050782 and the Phileona Foundation (KLM). The authors are grateful for resources provided by the University of Minnesota Supercomputing Institute and the Affymetrix core. We also thank Carolyn M. Meyer, Amber N. Leiran, Liliana Tobon, Daniella Machado, and Julie Ermer for their technical assistance and Jennifer Lessard for assistance with graphics. Finally, we thank the study participants without whom this study would not be possible.

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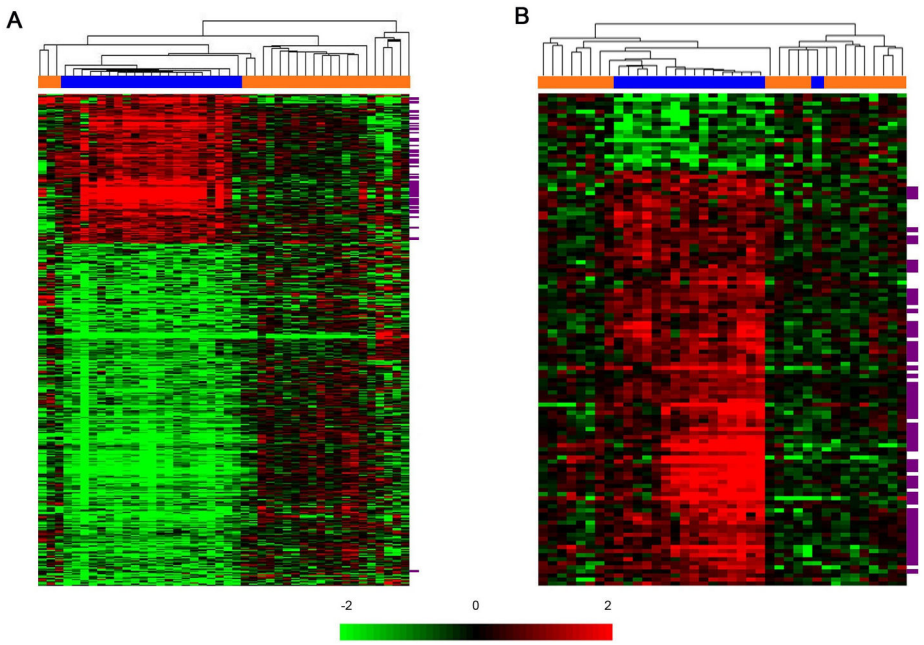

Figure 1. Gene Expression Profiles in Two Independent Sjögren's Syndrome Cohorts

Each row represents a single transcript and each column represents a single subject. SS cases (blue) and controls (orange) are indicated along the top of each cluster diagram and above each column of expression data. Horizontal bars on the right of each diagram indicate IFN-inducible genes (purple), previously defined by direct *in vitro* stimulation experiments or other data from the literature[22]. Log_2 transformed ratios of individual expression values divided by the mean of the controls were calculated for each transcript. These values were used in hierarchical clustering analyses. Relative intensities are indicated for overexpressed (red) and underexpressed (green) transcripts. (A) Differentially expressed transcripts (n=425) for Cohort 1. (B) Differentially expressed transcripts (n=120) for Cohort 2.

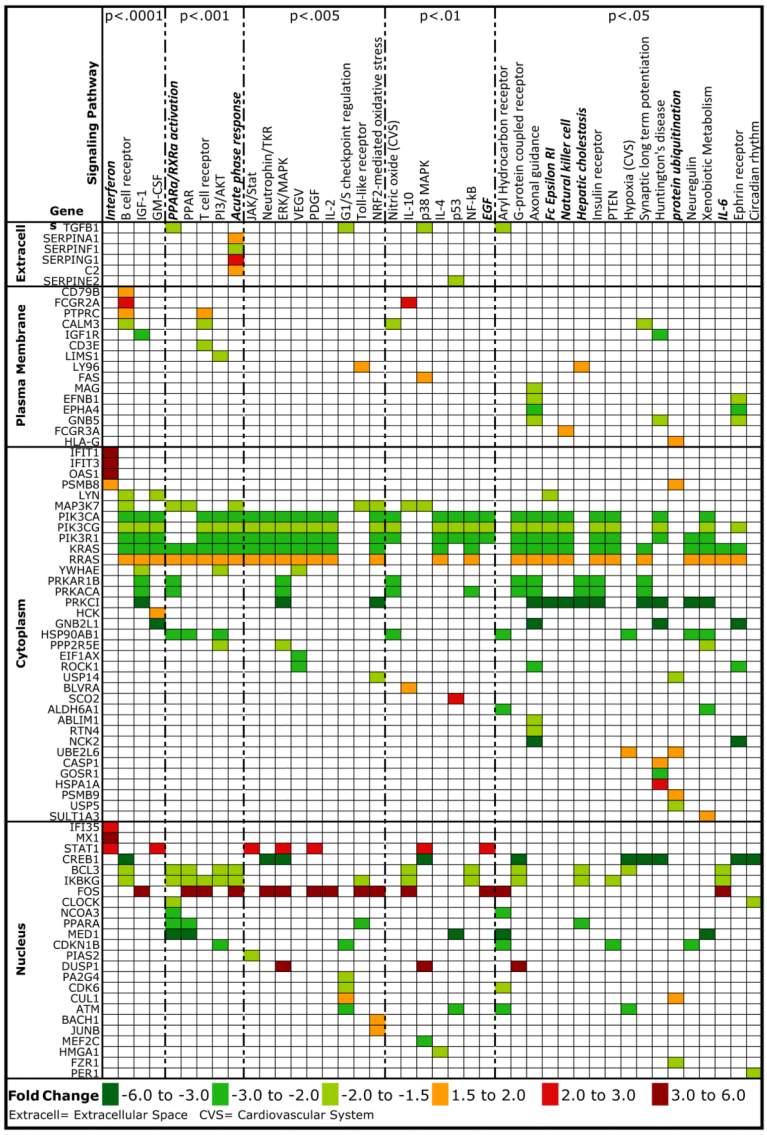

Figure 2. Summary of statistically significant canonical pathways identified through IPA

Canonical pathways are listed across the top from left to right in order of statistical significance in Cohort 1 with P value ranges indicated. Pathways indicated in bold italics represent those showing significance in both Cohorts 1 and 2. The left most column lists differentially expressed genes initially grouped by structural category to show cellular localization (extracellular, plasma membrane, cytoplasm, or nucleus). The genes within each of the 4 structural categories are further organized by ranking each gene according to initial occurrence in the most significant canonical pathway as statistically ranked across the top from left to right. The color-coded boxes indicate the fold-change differences in mean expression levels for SS cases in Cohort 1 relative to controls.

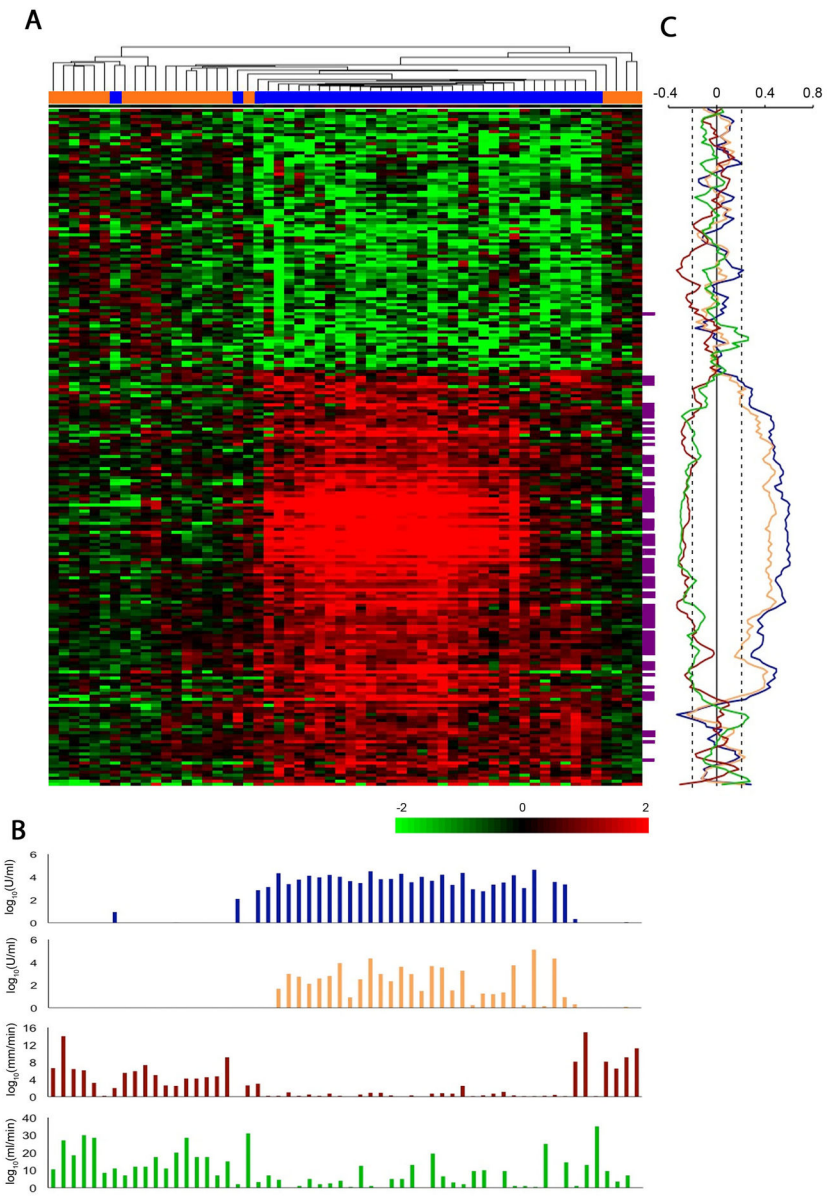

Figure 3. Correlation of clinical features with gene expression profiles in SS

(**A**) Hierarchical clustering analysis of 223 differentially expressed transcripts between 36 SS cases (blue) and 22 controls (orange) in Cohort 3. Color-coding is as described in Figure 1. (**B**) Bar graphs showing the distribution of measurements for anti-Ro/SSA (blue) and anti-La/SSB (gold) autoantibodies as measured by ELISAs, tear flow measurements as measured by Schirmer's Tests (ST; maroon) and whole unstimulated salivary flow (WUSF; green) for each individual in panel A. (**C**) Correlations between RNA transcript levels (rows) in panel A and clinical measurements of autoantibodies, tear flow, and salivary flow. Dashed lines indicate statistical significance thresholds (P=0.05) determined through permutation testing.

Table 1

Demographic and clinical data for SS cases

	Cohort 1	Cohort 2	Cohort 3[a]
Total subjects (n)	21	17	36
Sample type	PBMCs [b]	WB [b]	WB [b]
GeneChip version	U95A	U133A	U133A
Number of transcripts assayed	12,626	22,283	22,283
Demographic			
Mean Age ± s.d. (range)[c]	57 ± 11 (34–74)	63 ± 11 (44–80)	60 ±11 (34–80)
Female, n (%)	21 (100%)	16 (94%)	35 (97%)
Caucasian, n (%)	21 (100%)	17 (100%)	35 (97%)
Laboratory measurements			
Anti-Ro/SSA positive[d]	19 (90%)	16 (94%)	33 (92%)
Anti-La/SSB positive[d]	18 (86%)	13 (76%)	30 (83%)
WUSF[e], ≤1.5ml/15 minutes	20 (95%)	13 (76%)	29 (81%)
ST[e], ≤5mm/5 minutes	17 (81%)	12 (71%)	27 (75%)
Positive LSG biopsy[f]	2/2	3/3	5/6
Current Medications			
Anticholinergics	7 (33%)	6 (35%)	13 (36%)
NSAIDS	3 (14%)	5 (29%)	9 (25%)
Hydroxychloroquine	7 (33%)	5 (29%)	12 (33%)
Steroids	4 (19%)	5 (29%)	8 (22%)

[a] Cohort 3 includes 19 subjects from Cohort 1 (re-drawn) and all 17 subjects from Cohort 2

[b] PBMCs = peripheral blood mononuclear cells, WB = whole blood

[c] Age in years, S.D. = standard deviation

[d] Data were obtained from medical records and ELISA testing of samples obtained for this study

[e] WUSF = whole unstimulated salivary flow, ST = Schirmer's test

[f] Labial salivary gland (LGS) biopsy data were obtained from medical records, indicated as number positive/number with available data

Table 2

Top 20 most significant biological function categories identified through IPA

Biological functions
Cell Death
Cellular Growth and Proliferation
Immune and Lymphatic System Development and Function
Tissue Morphology
Gene Expression
Hematological System Development and Function
Cellular Development
Immune Response
Cancer
Cell Cycle
Immunological Disease
Inflammatory Disease
Organismal Injury and Abnormalities
Hematological Disease
Cell Signaling
Connective Tissue Disorders
Skeletal and Muscular Disorders
Cell Morphology
Organismal Development
Post-Translational Modification

Table 3

Independent replication of differentially expressed genes

Gene name	Gene Symbol	Cohort 1		Cohort 2	
		FC	p-value	FC	p-value
2′,5′-oligoadenylate synthetase 1, 40/46kDa	OAS1	3.44	1.05×10^{-07}	3.11	1.66×10^{-04}
2′-5′-oligoadenylate synthetase-like	OASL	3.05 3.52	5.6×10^{-07} 3.91×10^{-08}	2.43 2.10	1.31×10^{-05} 5.29×10^{-07}
Bone marrow stromal cell antigen 2	BST2	1.53	6.15×10^{-04}	1.54	1.08×10^{-04}
Carcinoembryonic antigen-related cell adhesion molecule 1	CEACAM1	2.05	8.22^{-04}	1.62	1.64×10^{-04}
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	2.00	9.29×10^{-09}	2.16	9.96×10^{-08}
Fc fragment of IgG, high affinity Ia, receptor for (CD64)	FCGR1A	2.05	1.43×10^{-04}	1.88	1.90×10^{-04}
Guanylate binding protein 1, IFN-inducible, 67kDa	GBP1	2.03	9.97×10^{-06}	1.96	4.13×10^{-04}
Hexokinase 3 (white cell)	*HK3*	1.54	2.18×10^{-04}	1.84	1.78×10^{-06}
Hypothetical protein FLJ38348	*FLJ38348*	1.74	1.93×10^{-04}	2.28	4.60×10^{-06}
IFN induced transmembrane protein 1 (9–27)	IFITM1	1.72	2.87×10^{-09}	1.94	2.91×10^{-11}
IFN induced transmembrane protein 3 (1-8U)	IFITM3	2.04	1.04×10^{-07}	1.88	2.95×10^{-09}
IFN regulatory factor 7	IRF7	2.22	1.98×10^{-07}	2.13	5.57×10^{-06}
IFN stimulated gene 20kDa	ISG20	1.56	1.33×10^{-06}	1.58	3.38×10^{-05}
IFN alpha-inducible protein (clone IFI-15K)	G1P2	3.19 3.10	7.87×10^{-09} 1.92×10^{-08}	4.98 3.14	9.55×10^{-06} 2.31×10^{-06}
IFN alpha-inducible protein 27	IFI27	24.39	1.31×10^{-07}	7.27	4.08×10^{-05}
IFN gamma-inducible protein 16	IFI16	1.54	2.73×10^{-04}	1.57	3.36×10^{-04}
IFN gamma-inducible protein 30	IFI30	1.56	2.47×10^{-07}	1.74	4.65×10^{-06}
IFN-induced protein 35	IFI35	2.42	1.78×10^{-09}	2.33	1.47×10^{-06}
IFN-induced protein 44	IFI44	3.81	2.63×10^{-08}	3.17	1.67×10^{-04}
IFN-induced protein with tetratricopeptide repeats 1	IFIT1	4.04	8×10^{-07}	6.58	4.31×10^{-05}
IFN-induced protein with tetratricopeptide repeats 2	IFIT2	2.95	6.19×10^{-04}	1.83	1.48×10^{-04}
Myxovirus resistance 1 IFN-inducible protein p78	MX1	3.68	9.94×10^{-08}	4.01	1.02×10^{-05}
Myxovirus (influenza virus) resistance 2	MX2	2.30	1.16×10^{-08}	1.76	2.52×10^{-07}

Gene name	Gene Symbol	Cohort 1		Cohort 2	
		FC	p-value	FC	p-value
Phospholipid scramblase 1	PLSCR1	1.87	7.29×10^{-06}	2.19	4.87×10^{-04}
Proteasome subunit, beta type, 9	PSMB9	1.59	3.96×10^{-07}	1.67	1.31×10^{-07}
S100 calcium binding protein A11 (calgizzarin)	*S100A11*	1.77	3.03×10^{-07}	1.61	3.58×10^{-04}
SCO cytochrome oxidase deficient homolog 2 (yeast)	*SCO2*	2.10	1.7×10^{-05}	1.61	9.14×10^{-05}
Secreted and transmembrane 1	SECTM1	1.59	8.79×10^{-05}	1.57	2.24×10^{-05}
Serine/cysteine proteinase inhibitor clade A member 1	*SERPINA1*	1.62	1.75×10^{-04}	1.56	3.79×10^{-06}
Serine/cysteine proteinase inhibitor clade G member 1	SERPING1	2.42	1.16×10^{-05}	2.48	8.45×10^{-07}
Signal transducer and activator of transcription 1, 91kDa	STAT1	2.09 2.28	6.64×10^{-06} 2.53×10^{-09}	1.75 1.66	8.14×10^{-04} 4.95×10^{-05}
SP110 nuclear body protein	SP110	1.56	2.41×10^{-05}	2.13	1.95×10^{-06}
Spermidine/spermine N1-acetyltransferase	*SAT*	1.52	3.41×10^{-04}	1.57	1.75×10^{-04}
Tryptophanyl-tRNA synthetase	WARS	1.60	1.76×10^{-04}	1.79	1.60×10^{-06}
Tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	2.06	5.47×10^{-06}	1.69	7.86×10^{-04}
Tumor necrosis factor receptor superfamily, member 6	TNFAIP6	1.57	1.66×10^{-04}	2.03	2.19×10^{-04}
Ubiquitin-conjugating enzyme E2L 6	UBE2L6	1.84	2.52×10^{-07}	1.79	9.71×10^{-08}
Zinc finger protein 91 (HPF7, HTF10)	*ZNF91*	−2.41	2.48×10^{-06}	−1.60	6.85×10^{-04}

FC = fold change

Genes not known to be inducible by IFN are italicized

Table 4

Transcripts encoding selected IFNs and IFN pathway regulators

		Cohort 1		Cohort 2	
Gene title	**Gene Symbol**	**p-value**	**Fold Change**	**p-value**	**Fold Change**
interferon (alpha, beta and omega) receptor 1	IFNAR1	0.1375	1.173	0.9081	1.019
interferon (alpha, beta and omega) receptor 2	IFNAR2	0.5259	1.055	0.3731	−1.207
interferon (alpha, beta and omega) receptor 2	IFNAR2	0.0915	−1.485	0.6485	−1.053
interferon gamma receptor 1	IFNGR1	0.1253	1.201	NA	NA
interferon gamma receptor 1	IFNGR1	0.3864	1.141	0.2215	1.119
interferon gamma receptor 2	**IFNGR2**	**0.002286**	**1.325**	**0.0067**	**1.234**
interferon regulatory factor 1	**IRF1**	**4.90E-05**	**1.292**	0.3639	1.081
interferon regulatory factor 2	**IRF2**	0.3061	1.089	**0.002031**	**−1.51**
interferon regulatory factor 2	**IRF2**	NA	NA	**0.006993**	**−2.218**
interferon regulatory factor 2 binding protein 1	IRF2BP1	0.2456	−1.266	**0.01611**	**−1.522**
interferon regulatory factor 3	IRF3	0.849	−1.016	0.2677	1.072
interferon regulatory factor 4	IRF4	0.8907	1.014	0.7614	−1.084
interferon regulatory factor 4	IRF4	0.5434	1.115	0.5474	1.165
interferon regulatory factor 4	IRF4	0.4046	−1.373	0.5748	−1.053
interferon regulatory factor 5	**IRF5**	0.8622	−1.031	**1.01E-04**	**1.484**
interferon regulatory factor 5	**IRF5**	0.5132	1.101	**9.95E-05**	**1.398**
interferon regulatory factor 5	IRF5	NA	NA	0.07417	1.214
interferon regulatory factor 6	**IRF6**	0.09311	1.293	**0.03743**	**−1.152**
interferon regulatory factor 7	**IRF7**	**5.57E-06**	**2.134**	**1.98E-07**	**2.223**
interferon regulatory factor 8	IRF8	0.07352	−1.147	NA	NA
interferon, alpha 1	IFNA1	0.5678	−1.141	0.1016	1.405
interferon, alpha 1	IFNA1	0.1796	1.369	**9.27E-04**	**−2.263**
interferon, alpha 14	IFNA14	0.2596	1.167	0.2739	−1.232
interferon, alpha 17	IFNA17	0.6196	1.043	NA	NA
interferon, alpha 2	IFNA2	0.1041	−1.708	0.2332	−1.422
interferon, alpha 21	IFNA21	0.4626	−1.204	0.3308	−1.273

Gene title	Gene Symbol	Cohort 1		Cohort 2	
		p-value	Fold Change	p-value	Fold Change
interferon, alpha 4	IFNA4	0.5041	1.212	0.5648	−1.111
interferon, alpha 4	IFNA4	0.7891	−1.078	NA	NA
interferon, alpha 4	IFNA4	0.4413	1.092	NA	NA
interferon, alpha 5	IFNA5	0.7628	−1.048	0.1914	−1.3
interferon, alpha 5	IFNA5	NA	NA	0.4344	−1.257
interferon, alpha 6	**IFNA6**	0.5977	1.197	**0.00801**	**−1.498**
interferon, alpha 8	IFNA8	0.3165	1.338	0.08078	−1.579
interferon, beta 1, fibroblast	IFNB1	0.1933	−1.194	0.3853	−1.224
interferon, gamma	IFNG	0.2801	−1.106	0.3427	1.266
interferon, gamma	IFNG	NA	NA	0.2063	1.224
interferon, gamma	IFNG	NA	NA	0.9056	1.034
interferon, omega 1	IFNW1	0.9864	1.004	0.6354	1.087
interleukin 6 (interferon, beta 2)	IL6	0.6588	−1.088	0.7582	−1.108
interferon-related developmental regulator 2	**IFRD2**	**0.006021**	**−1.362**	NA	NA
interferon, alpha 16	IFNA16	NA	NA	0.2449	−1.279

Significant values are shown in bold (*P*-value<0.05)

NA = Not Analyzed; indicates missing data where probe set was not present on the GeneChip version used in either Cohort 1 or 2. Specific probesets for these results are provided in the Supplementary Tables.