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Up-regulated gene expression in the conjunctival epithelium of patients with Sjögren’s syndrome

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Abstract

\textbf{Purpose.} To elucidate the pathogenesis of ocular surface abnormalities in patients with Sjögren’s syndrome (SS) by comparing global gene expression patterns in conjunctival epithelial cells from normal individuals and SS patients.

\textbf{Methods.} The study population consisted of 56 subjects (26 SS patients and 30 normal volunteers). RNA extracted from their conjunctival epithelial cells was subjected to introduced amplified fragment length polymorphism (IAFLP), a competitive PCR-based gene expression assay, to measure gene expression in the 56 samples against 931 genes. Data were analyzed by two-dimensional clustering analysis and discriminant analysis. Disease-related genes were identified and the feasibility of gene expression-based diagnosis of SS was examined.

\textbf{Results.} Two-dimensional clustering- and discriminant analysis clearly distinguished between SS patients and normal subjects. Of 931 genes tested, 34 were significantly up-regulated and 12 were significantly down-regulated in SS ($p < 0.05$). Up-regulated genes included kalikrein 7 ($\times 15.8$) and small proline-rich protein 2A ($\times 9.6$), markers for the terminal differentiation of epidermis, and the inflammation-related genes HLA-DR and IL-6. Monokine-induced-by-gamma-interferon, i.e. c-fos, fibronectin, amphiregulin, defensin beta 2, and keratin 16, -6b and -6c were also up-regulated. Among the 12 down-regulated genes, interferon-gamma receptor 1 was most notable ($\times 1/27.3$).

\textbf{Conclusions.} The up-regulated expression of keratin 6 and -16, small proline-rich protein 2A, and kalikrein 7 in the conjunctival epithelium of SS patients suggests an anomalous keratinization pattern. Epithelial thickening may be due to amphiregulin and/or c-fos-stimulated cell cycle progression. The up-regulation of monokine-induced-by-gamma-interferon, HLA-DR, keratin 6b, -6c, and -16 suggests that in SS, interferon-gamma may play an important role in the altered gene expression in the conjunctival epithelium.

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\textbf{Keywords:} Sjögren’s syndrome; gene expression profiling; introduced amplified fragment length polymorphism; keratinization; conjunctival epithelium; interferon gamma

1. Introduction

Sjögren’s syndrome (SS) is an idiopathic autoimmune disease that affects the lacrimal and salivary glands. Clinical findings on the ocular surface of SS patients include aqueous tear deficiency, superficial punctate erosions on both the cornea and conjunctiva, and sometimes corneal filaments, resulting in pain, foreign body sensation, dryness, and asthenopia. Infiltrating lymphocytes impair the structure and function of lacrimal glands (Fox et al., 1985; Hikichi et al., 1993) and the resulting depleted tear secretion leads to severe dryness of the ocular surface mucosa. Lymphocyte infiltration into the substantia propria of the conjunctiva (Raphael et al., 1988; Rivas et al., 1992; Pflugfelder et al., 1997) and the up-regulated expression of the immune-modulator molecules HLA-DR and ICAM-1, and of the cytokines IL-6 and IL-1 on the conjunctival epithelial cells have been reported (Jones et al., 1994; Pflugfelder et al., 1997; Jones et al., 1998; Brignole et al., 2000). These observations led to the hypothesis that decreased tear secretion and inflammation play a role in the pathogenesis of ocular surface anomalies in SS. An understanding of this pathogenesis is critical for the development of treatments to alleviate
the symptoms and possible complications of patients with SS and other tear-depleted ocular surface diseases.

Assessment and comparison of gene expression changes on a genome-wide scale is a powerful tool for understanding the pathophysiology of diseases. Although various micro-arrays can be obtainable through commercial sources, there are no arrays that harbor the entire set of human genes and the gene repertoires on commercial arrays have apparent inclinations toward genes for major diseases in major organs. For the molecular descriptions of diseases in ocular surface, we have collected expressed sequence tags (ESTs) from ocular surfaces (Nishida et al., 1996) including, more recently, the conjunctiva (Dota et al., 2001). A combination of thus obtained sequence information and our originally developed PCR based transcript quantitation method, introduced amplified fragment length polymorphism (iAFLP), (Kawamoto et al., 1999), allows the altered expression of normally very active genes in ocular surface to be monitored from tiny amounts of biopsy specimens. In our quest for a molecular description of ocular surface diseases we compared gene expression patterns in conjunctival epithelial cells from normal subjects and patients with SS. We also considered the feasibility of gene expression-based diagnosis of this disease.

2. Materials and methods

2.1. Subjects

The study population consisted of 56 subjects. These were 26 SS patients (1 male, 25 females; mean age 62.6 ± 10.4 years) and 30 normal volunteers (17 males, 13 females; mean age 32.4 ± 7.8 years). Prior informed consent was obtained from all subjects after a detailed explanation of the procedures, in accordance with the tenets of the Declaration of Helsinki.

A diagnosis of SS was made when the case satisfied the criteria of Fox et al. (1986). The normal volunteers were confirmed to be free of biomicroscopic abnormalities of the cornea, conjunctiva, and meibomian glands, lacrimal drainage problems, signs of dry eye, and a history of contact lens wear.

2.2. Brush cytology and RNA extraction

After topical anaesthesia with 0.4% oxybuprocaine eye drops, conjunctival epithelial cells around the temporal and nasal area of the bulbar conjunctiva were collected using a sterile nylon thread brush (Cytobrush®, Medscand Medical AB, Malmö, Sweden) according to the method of Tsubota et al. (1990). The brush was then immersed for 5 min at room temperature in 300 μl of acid phenol with guanidine isothiocyanate (TRIZOL® reagent, Invitrogen Corp., Carlsbad, CA, USA). Total RNA was extracted from the harvested epithelial cells and dissolved in 10 μl of distilled water according to the manufacturer’s guidelines. For quantitation and an integrity check, 1 μl of the RNA solution was electrophoresed on 1% agarose gel. The gel was stained with an RNA staining reagent (SYBR® Green II RNA gel stain, Molecular Probes Inc., Eugene, OR, USA) and 18S and 28S ribosomal RNA band fluorescence was quantitated using a luminescent image analyzer (LAS1000, Fuji Film Medical Systems Inc., Stamford, CT, USA). Spectrophotometrically quantitated total RNA from a human cell line (in this case the human epithelial cell HepG2) was used as the RNA standard for absolute quantitation. Residual RNA was stored in a −80°C freezer until use.

2.3. Gene expression analysis by iAFLP

We used the iAFLP method of Kawamoto et al. (1999). The integrity of cDNA synthesis, from the extracted sample RNA, was ascertained by using a, non-commercial, vector primer (pUC 19-based vector primer) having a T-tail as a primer for mRNA’s poly(A) (Okubo et al., 1992). Use of this vector primer facilitated the study of very small amounts of cDNA. Extracted total RNA (about 100 ng) was annealed with the pUC 19-based vector primer (5 ng) in a total volume of 10 μl. After 3-min heat denaturation at 70°C, 10 μl of 2× reaction mixture were added to yield a final concentration of 0.5 mM dNTP, 1× RT buffer (supplied with SuperScriptII), 15 mM DTT, and 0.1 U/μl of reverse transcriptase (SuperScriptII, Invitrogen). This was then incubated at 42°C for 60 min. For second-strand synthesis, 130 μl of a second-strand reaction mixture was added to yield a final concentration of 0.33 mM dNTP, 2.7 mM DTT, 1× E. coli ligase buffer (supplied with E. coli ligase), 0.27 U/μl of DNA polymerase I (Invitrogen), 0.13 U/μl of E. coli ligase (Invitrogen), and 0.013 U/μl of E. coli RNase H (Invitrogen). After purification by phenol-chloroform extraction and ethanol precipitation, the resultant cDNA was dissolved in 20 μl of distilled water and 2.5 μl aliquots of the solution were stored for electrophoresis.

For cDNA digestion, 2 μl of 10× NEB3 buffer (supplied with MboI) and 5 U of MboI restriction enzyme (New England Biolabs Ltd, Hertfordshire, UK) were added. This was followed by 60-min incubation at 37°C, and heat inactivation at 70°C for 20 min. A 2.5 μl aliquot of the digested cDNA was electrophoresed on an agarose gel along with stocked undigested cDNA to check the quality of cDNA synthesis and MboI digestion. From each of the conjunctival epithelial samples only approximately 100 ng of total RNA was extracted. This is not a sufficient amount for the subsequent analysis of hundreds of genes. Therefore the cDNA library from each sample was amplified by PCR. A T7-1400 adaptor once ligated to the cDNA library was used as a template for PCR amplification.

To prepare each template cDNA for PCR amplification, 100 p moles of T7-1400 adaptor (for sequences, see
Oligomers section), 10 n moles of ATP, 100 U of T4 DNA ligase (New England Biolabs), 10 x T4 ligase buffer (supplied with T4 DNA ligase, final conc.: 1 x ) were added to the MboI digested cDNA from each sample to a final volume of 40 µl, and incubated at 16°C for 3 hr. Using 2 µl of this mixture as a template, PCR was performed in 50 µl with a final concentration of 1 x Platinum Taq buffer (supplied with Platinum Taq DNA polymerase), 0.05 U/µl of Platinum Taq DNA polymerase (Invitrogen), 2 mM magnesium chloride, 0.2 mM dNTP, and 10 p moles each of primers Bam7T and T7(23). The thermal PCR conditions with a 3-min pre-incubation at 94°C and final extension at 72°C for 10 min, were 30 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min.

A small aliquot of the PCR product was electrophoresed on a 1% agarose gel to confirm appropriate amplification. MboI was added to this reaction for cleavage of amplified cDNA and the reaction mixture was incubated at 37°C for 60 min, followed by heat inactivation at 70°C for 20 min. After purification by phenol-chloroform extraction and ethanol precipitation, the precipitate was dissolved in 63 µl of distilled water.

The iAFLP method used here can directly compare gene expression among six samples at a time; a comparison of more than six samples was achieved by preparing a reference sample as is common in 2-colour DNA microarray analysis. Although a mixture of various individual cell lines is commonly used as a reference in DNA microarrays, we adopted a mixture of all the samples. Using a mixture of all the samples as a reference prevents data loss, when measuring its amount by fluorescence, caused by either peak saturation or the peak level falling below the background noise. The reference sample (approx. 600 µl) was prepared by pooling 10 µl of amplified cDNA from all samples. Five samples and a reference sample were mixed to form a template for profiling PCR. The sample data could then be normalized by dividing by the respective value of the reference sample allowing for the comparison of more than six samples.

Reference samples and each amplified cDNA sample were ligated at 16°C for 3 hr with their corresponding LP adapters (for sequences, see Oligomers section). LP adapters were used to induce a size difference in the PCR amplified cDNA fragments. This size difference was subsequently used to resolve differences in gene expression between individual samples by electrophoresis. The first cDNA sample was ligated to an LP 40 adaptor molecule, the second sample to an LP 43, the third to an LP 46, the fourth to an LP 49 and the fifth to an LP 52. The subsequent samples (numbers 6–56) were then ligated (in groups of five) to LPs 40–52, respectively. The reference samples were ligated to an LP 55 adaptor. The cDNA samples were in final volumes of 100 µl whereas the reference samples were in final volumes of 1200 µl. Each sample contained 1 U/µl of T4 DNA ligase, 1 x T4 DNA ligase buffer, and 200 p moles/µl of LP adaptor. An even mixture of five individual samples and one reference sample served as the template for profiling PCR; a total of 12 templates were made from 56 independent samples from 56 study participants. To measure relative transcript abundance among samples in each template, PCR was performed in 10 µl volumes using 0.05 µl of profiling template with a final concentration of 1 x Platinum Taq buffer, 0.05 U/µl of Platinum Taq polymerase, 2 mM magnesium chloride, and 0.2 mM dNTP, 0.2 p moles/µl each of gene-specific primers and F-T7(25). To measure the ratio of total cDNA amounts among sample cDNAs in each profiling template, a vector portion of cDNA was amplified with primers Anti-Bam and F-T7(25) using the above PCR reaction mixture.

The PCR product (0.4 µl) was mixed with 1.6 µl of formamide-loading buffer containing 1/4 volume of TAMRA-labeled size marker (GeneScan 350 Tetramethylrhodamine (TAMRA), Applied Biosystems, Foster City, CA, USA). The TAMRA-labeled size maker fluoresces at 572 nm under a 555 nm light, and was used to check the validity of the PCR product. After 2-min denaturation at 94°C, 0.5–1.0 µl of the mixture was electrophoresed on 10% acrylamide gel containing 6 M urea with a 15 cm well-to-detector distance. An auto-sequencer (ABI PRISM 377XL, Applied Biosystems) and fragment analysis software (Genescan, Applied Biosystems) were used to quantitate each dye-labeled amplified fragment. Each fragment was resolved by virtue of the size of its attached LP adaptor.

2.4. Oligomers

The following iAFLP oligomers were used:

LP40: 5'-ACGACTCACTATAGGGAGATTTCACTCAGTCAGGAT
LP43: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP46: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP49: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP52: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP55: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP57: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP59: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP61: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP63: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP65: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP67: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP69: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP71: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP73: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP75: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP77: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP79: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP81: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP83: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP85: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP87: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP89: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP91: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP93: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP95: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP97: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT
LP99: 5'-ACGACTCACTATAGGGGATTTCACTCAGTCAGGAT

P means kination of the 5' end and NH2 means termination of the 3' end by an amino linker. NH1400 was annealed with each iAFLP oligomer or T7-1400, using an equimolar amount to form each LP adaptor or T7-1400 adaptor, respectively.
PCR primers were as follows:

Bam7T: 5'-GACGCGATCCAAGCTTGCAATGCTGCTG
T7(23): 5'-TAATACGACTCACTATAGGGAGA
5'-Fam-labeled T7(25): 5'-F-ACGACTCACTATAGGGAGG
Anti-Bam: 5'-GATCCAGGGCATGCAAGCCTTG

To design each gene-specific primer, the gene sequence between poly(A) and the most 3' GATC sequence was obtained from the Bodymap database (http://bodymap.ims.u-tokyo.ac.jp/) or the Genbank database (http://www.ncbi.nlm.nih.gov/). Each gene-specific primer was designed on the 3' side of the GATC site as an anti-sense primer with an appropriate S value (length between the G base of the most 3' GATC sequence and the 3' end of the primer). Using this S value, the electrophoresis results were validated by a custom-made program.

The oligomers were custom-made by Gensetoligo (Paris, France) except for the 5'-Fam-labeled T7(25), which was from Applied Biosystems.

We synthesized PCR primers from 733 genes previously shown to be active in the human conjunctival epithelial cells (Dota et al., 2001). The sequence of each 3'-directed EST cluster was taken from the BodyMap database (http://bodymap.ims.u-tokyo.ac.jp/) and subjected to primer design for each gene by our custom made program. When compared with other 51 3'-directed cDNA libraries in the Bodymap database, the 733 genes were shown to contain 24 genes commonly expressed in all tissues and 26 genes that are preferentially or specifically expressed in the body surface epithelium. Furthermore, we found that 25 genes were unique to the conjunctival epithelium as compared to other cDNA libraries. We also designed primers for inflammation mediators relevant to SS, and for genes that are selectively induced in other epidermal diseases. Altogether, we synthesized 931 primers representing 600 known and 331 anonymous genes.

2.5. Data analysis

Firstly, the peak values from each fluorescent dye-labeled amplified fragment which corresponded to authentic gene products in each sample, as judged by the custom made program, were normalized. Normalization was achieved by dividing the peak values of each gene product by their respective reference value and by each samples total amount of cDNA. Secondly, the resultant gene expression matrix describing the amount of differing genes by the number of samples, was then subjected to the statistical analyses, two-dimensional cluster analysis and discriminant analysis.

Cluster analysis was performed with freely available Cluster software (http://rana.lbl.gov/, Eisen et al., 1998) by following its attached guidelines. Cluster analysis is a multivariate statistical test which accomplishes the task of partitioning a set of objects (in this case gene expression profiles from normal subjects and those suffering from SS) into relatively homogeneous subsets based on the inter-object similarities. The results of cluster analysis were visualized using TreeView software (http://rana.lbl.gov/).

Significance between the gene expression levels in normal subjects and those suffering from SS were calculated by a standard statistical method, the two-tailed Welch test which compares the mean values between two groups which differ in variance.

Discriminant analysis, using the 'Mahalanobis distances', was performed to assess the feasibility of predicting and diagnosing SS by gene-expression profiling alone. Discriminant analysis is a procedure for identifying such relationships between qualitative criterion variables, such as SS or normal groups and quantitative predictor variables, such as gene type. Discriminant analysis was preformed with a commercially available statistical package (SAS, SAS Institute Inc., Cary, NC, USA). Mahalanobis distances were calculated from each sample to the center of an actual SS group (Mahalanobis distance 1) and an actual normal group (Mahalanobis distance 2) and the result plotted on a scattergram.

3. Results

On average, 100 ng of total RNA was recovered from the 26 SS patients and 30 normal volunteers. All sample RNAs were individually converted into cDNA, quantitated, adapted, and used for quantitative amplification of the selected 931 gene sequences for gene expression profiling by iAFLP. Of the 931 primers tested, 578 yielded products in expected sizes, suggesting that the signals represent concentrations of target transcripts. Other primers failed to amplify specific products due to errors in sequence information or low-level expression of the target genes.

Epithelial cells from SS patients and normal subjects yielded different gene expression patterns. Two areas of significant change in either the up- or down-regulation of gene expression in samples from SS patients were found by cluster analysis based sample similarity. One such area incorporated 53 genes including 34 genes which were significantly up-regulated (Fig. 1(B), Table 1(A)). Another area contained 15 genes 12 of which were significantly down-regulated (Fig. 1(B), Table 1(B)). Within these two areas the largest variations in gene expression were displayed by KLK7 (×15-8), monokine induced by gamma interferon (×9-9) and small proline-rich protein 2A (×9-6) which were remarkably up-regulated and IFN-gamma (×1/27.3) receptor 1 which was remarkably down-regulated in SS. Some of the remaining genes also showed differences in gene expression however these were more evenly distributed between SS and normal subjects and therefore did not indicate any relevance to disease status.
Fig. 1. Hierarchical clustering of gene expression data from iAFLP method. The height of the resulting hierarchical tree (dendrogram) from cluster analysis represents the similarity between sample pairs, the highest pair being the least similar in gene expression. Each row represents an individual gene and each column an individual sample. Data were log-transformed (base2) and centered in row-direction (gene) by subtracting the median observed value (log-space). Data are depicted according to the colour scale (log-space) shown at top left. Gray data values indicate the row electrophoresis data were the cutoff value. (A) Whole image of two-dimensional hierarchical clustering of 578 genes across 56 samples. Upper hierarchical tree is colour-coded as shown at the top right. Areas demarcated in light blue and yellow show genes whose expression was markedly different between samples from SS patients and those from normal subjects (blue, down-regulated; yellow up-regulated). (B) Zoomed image of portion of (A). Areas demarcated in light-blue and yellow correspond to the areas demarcated in the same colour in (A), respectively. Some of the significantly down-regulated and up-regulated genes are marked at the right side. Upper hierarchical tree and samples are colour-coded as shown at the top right.

Clustering analysis, displayed as hierarchical trees, provides a distinctly different pattern for most SS patients and normal volunteers (Fig. 1(A) and (B)). Of the 26 SS patients, 23 clustered in the SS group and 3 in the normal group. None of the 30 normal subjects clustered in the SS group. Therefore, the diagnosis of SS, solely based on gene expression, was 100% specific with 90.9% sensitivity.

The 34 genes that exhibited a clearly different expression pattern in SS- and normal samples (Table 1(A)) were subjected to discriminant analysis using the Mahalanobis distances and the result was plotted on a scattergram (Fig. 2). A sample with a high ratio of Mahalanobis distance 1 to Mahalanobis distance 2 indicated that its gene expression pattern was more similar to normal subjects than to SS patients. The consequence of this analysis was successful discrimination at 100% sensitivity and 100% specificity.

4. Discussion

Immunopathological studies suggest that in SS, lymphocytes infiltrating the lacrimal and salivary glands (Fox et al., 1985; Hikichi et al., 1993) recognize specific auto-antigens. Lymphocytic infiltration leads to impairment of the glandular structure and function and can result in severe dry eye and dry mouth. Histological abnormalities on the ocular surface of SS patients include a decrease in the number of goblet cells, squamous cell metaplasia, and keratinization and T lymphocyte infiltration of the conjunctival epithelial layer (Raphael et al., 1988; Rivas et al., 1992; Pflugfelder et al., 1997). In addition, the expression of the immune-regulation molecules HLA-DR and ICAM-1 and of several cytokines on conjunctival epithelial cells are up-regulated (Jones et al., 1994; Pflugfelder et al., 1997;
<table>
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<th>Definition</th>
<th>Expression ratio (SS/normal)</th>
<th>Welch test (two-tailed)</th>
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<td>Kalikrein 7</td>
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</tr>
<tr>
<td>GS1681</td>
<td>CGI107 protein (LOCS1012)</td>
<td></td>
<td>2.06</td>
<td>1.3 × 10(^{-5})</td>
</tr>
<tr>
<td>GS1465</td>
<td>None</td>
<td>None</td>
<td>1.95</td>
<td>4.4 × 10(^{-3})</td>
</tr>
<tr>
<td>GS1465</td>
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<td>None</td>
<td>1.90</td>
<td>1.7 × 10(^{-3})</td>
</tr>
<tr>
<td>GS5113</td>
<td>SOD2</td>
<td>SOD-2 manganese superoxide dismutase</td>
<td>1.85</td>
<td>2.1 × 10(^{-3})</td>
</tr>
<tr>
<td>GS6513</td>
<td>MUC4</td>
<td>Mucin 4</td>
<td>1.83</td>
<td>1.9 × 10(^{-3})</td>
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<tr>
<td>GS6911</td>
<td>None</td>
<td>None</td>
<td>1.79</td>
<td>4.6 × 10(^{-4})</td>
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<tr>
<td>GS6262</td>
<td>GABRB</td>
<td>Gamma-aminobutyric acid (GABA) A receptor, pi</td>
<td>1.64</td>
<td>5.8 × 10(^{-3})</td>
</tr>
<tr>
<td>GS14627</td>
<td>SLC34A2</td>
<td>Solute carrier family 34 (sodium phosphate), 2</td>
<td>1.60</td>
<td>6.1 × 10(^{-4})</td>
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<tr>
<td>GS8304</td>
<td>KRT17</td>
<td>Keratin 17</td>
<td>1.50</td>
<td>8.9 × 10(^{-2})</td>
</tr>
</tbody>
</table>

| GS5524    | IFNG1       | Interferon gamma receptor 1                                                  | 27.35                        | 3.8 × 10\(^{-3}\)      |
| GS1633    | LOCV2482    | LOC2482                                                                      | 5.39                         | 2.2 × 10\(^{-5}\)      |
| GS2374    | APM2        | Adipose specific 2                                                           | 2.67                         | 2.3 × 10\(^{-7}\)      |
| GS6099    | FABP5       | Fatty acid binding protein 5                                                  | 2.58                         | 2.3 × 10\(^{-7}\)      |
| GS844     | COL1A2      | Collagen, type I, alpha 2                                                     | 2.57                         | 2.9 × 10\(^{-4}\)      |
| GS810     | MYC         | c-myc                                                                        | 2.28                         | 1.1 × 10\(^{-3}\)      |
| GS6785    | None        | None                                                                         | 1.89                         | 6.8 × 10\(^{-3}\)      |
| GS7563    | MSLN        | Mesothelin                                                                    | 1.81                         | 1.2 × 10\(^{-2}\)      |
| GS6856    | None        | None                                                                         | 1.77                         | 2.8 × 10\(^{-3}\)      |
| GS1468    | None        | FLJ10261                                                                     | 1.61                         | 1.3 × 10\(^{-4}\)      |
| GS1264    | AQP3        | Aquaporin 3                                                                   | 1.52                         | 1.6 × 10\(^{-5}\)      |
| GS2100    | MD3033      | Hematopoietic stem/progenitor cells protein MDS303                            | 1.41                         | 2.1 × 10\(^{-2}\)      |

Jones et al., 1998; Brignole et al., 2000). Previous investigations focused on tear volume depletion and inflammation in SS. However, as genes associated with other biological processes may be involved in the pathogenesis of SS, we performed a comprehensive gene expression study. We compared data on conjunctival cells obtained from patients with SS and normal individuals to identify genes involved in the pathogenesis of SS and to test the possibility that gene expression patterns can be used to predict or diagnose SS.

Cluster- and discriminant analyses confirmed that assessment of the gene expression pattern is a powerful tool in the diagnosis of SS. Discriminant analysis of the 34 up-regulated genes yielded correct discrimination between
samples from SS patients and normal subjects. Conventional methods to diagnose SS include lip- and lacrimal biopsy. The harvesting of conjunctival epithelial cells by brush cytology is less invasive and patients experience no pain, or only little discomfort that disappears within 30 min. As the prediction accuracy of our method matches that of conventional methods, we prefer our diagnostic method and suggest that upon improvements in the assays, it can be used in the clinical setting.

We identified 34 significantly up-regulated and 12 significantly down-regulated genes in SS. Of the up-regulated genes, four are related to keratinization: SPRR2A, keratin 6b, -6c, and -16. SPRR2A is a precursor protein of the cornified envelope and a terminal differentiation marker of skin keratinocytes (Hohl et al., 1995). Therefore, significant up-regulation of SPRR2A suggests keratinization of the conjunctival epithelium.

Keratin 6, -16, and -17 are thought to be markers of activated skin keratinocytes that are highly expressed in psoriasis; these genes were also up-regulated in SS. In organ-cultured normal skin keratinocytes, keratin 16 and -17 are induced by IFN-gamma or IL-1 beta, while in organ-cultured epithelial cells they are blocked by IL-1 receptor antagonist or anti-IL-1 antibody (Wei et al., 1999). This suggests that the induction of keratin 16 and -17 is not a direct response to IFN-gamma, but rather an indirect response mediated by IL-1 beta. Keratin 6 was induced when organ-cultured normal skin keratinocytes were treated with IL-1 (Komine et al., 2000). If these cellular responses occur in conjunctival epithelial cells, the up-regulation in SS of keratin 6, -16, and -17 may reflect activation, initially via IFN-gamma, through IL-1 beta.

KLK7, which is expressed in skin and other tissues (Yousef et al., 2000), is involved in intercellular adhesion molecule degradation in cell desquamation (Lundstrom and Egelrud, 1991) and was shown to be up-regulated in SS patients. This gene is also up-regulated in psoriasis (Ekholm and Egelrud, 1999) and is thought to serve as a marker for terminal epidermal differentiation (Ekholm and Egelrud, 2000). Therefore, as in the case of psoriasis skin scales, significant up-regulation of KLK7 may be responsible for the cell shedding in the conjunctival epithelium of SS patients. This, in turn, may result in punctate conjunctival erosions. Based on these considerations, we suggest that KLK 7 may play a crucial role in the pathogenesis of the persistent punctate conjunctival erosion in SS patients.

The up-regulation of IL-6 in SS patients found in this experiment supports the previous findings of Jones et al. (1994, 1998). However, the up-regulation of MIG and amphiregulin has not been documented previously. MIG, a cytokine secreted by monocytes, is induced by IFN-gamma (Liao et al., 1995). Langerhans cells and differentiated cells from monocytes found in conjunctival epithelium are most probably the source of this cytokine which exerts stimulative and chemotactic activity on activated lymphocytes (Liao et al., 1995). We posit that MIG is responsible for the conjunctival infiltration by T lymphocytes.

Amphiregulin is an autocrine-type growth factor that binds to the EGF receptor and affects cell proliferation. In skin keratinocytes, it facilitates cell proliferation and it is reportedly up-regulated in psoriasis (Piepkorn, 1996). Furthermore, thickening of the conjunctival epithelial layer in SS is similar in histological appearance to the thickening seen in psoriasis, suggesting that amphiregulin has a similar pathogenetic role in SS and psoriasis. Asano et al. (1997) reported that IFN-gamma induces amphiregulin expression in normal human bronchial epithelial cells. If this is true for conjunctival epithelium, the thickening of the epithelial layer in SS may be attributable to IFN-gamma-induced amphiregulin. In SS samples, c-fos, which is involved in cell cycle progression (Pui and Bird, 1994), was increased. Thickening of the epithelial layer may also be the result of enhanced cell proliferation attributable to up-regulation of this gene.

Fibronectin plays an important role in wound healing and is induced by IFN-gamma (Maheshwari et al., 1990). Up-regulation of this gene in SS may be the result of induction by IFN-gamma, and may be a physiological response to heal conjunctival punctate erosions.

Our study confirms earlier reports that HLA-DR is up-regulated in SS (Jones et al., 1994; Pflugfelder et al., 1997; Brignole et al., 2000). De Saint Jean et al. (1999) and Tsubota et al. (1999), who showed significant induction of this gene by IFN-gamma in the Chang conjunctival cell line via the NF-kB signaling pathway, also proposed that IFN-gamma is responsible for the up-regulated expression of HLA-DR in SS.

Defensin beta 2, a secretory protein with antimicrobial activity (Schröder and Harder, 1999), was also up-regulated in SS. This gene is not constitutively expressed, and it is potentiated by IL-1 beta. IFN-gamma or some inflammatory cytokine(s) may induce defensin beta 2 in SS.
IFN-gamma receptor 1 was significantly down-regulated in SS patients. This gene is ubiquitously expressed and is crucial for cellular responses to IFN-gamma. In psoriatic skin, it is down-regulated in the upper epidermis while in normal skin it is expressed throughout the epidermal layer (Scheuynis et al., 1992). Psoriatic keratinocytes manifest an attenuated response to IFN-gamma (Baker et al., 1988; Chen et al., 2000). The conjunctival epithelium may respond to chronic stimulation by IFN-gamma and down-regulate this gene, as is the case in the upper epidermis of psoriatic skin.

The gene, adipsic 2, was decreased in SS patients. The function of this gene is still unknown, but interestingly, we found that this gene was actively transcribed in corneal epithelial cells from the Bodymap database (34 clones/3485 clones corrected). The actively transcribed status of this gene in corneal epithelial cells and decreased expression in conjunctival epithelium of SS prompt us to speculate an important role for this gene in maintaining ocular surface integrity.

Fatty acid binding protein 5 is a keratinocyte protein that is highly up-regulated in psoriatic plaques (Madsen et al., 1992). This gene was reportedly induced by retinoic acid in vivo human skin (Larsen et al., 1994). Combined with the facts that retinoic acid is contained in tear fluid (Ubelis and MacRae, 1984) and that tear volume is reduced in SS, this gene may be decreased due to reduced supply of tear retinoic acid.

Collagen type I alpha 2, a major ECM component in tendon and ligaments, and c-myc, a gene involved in controlling cell proliferation and differentiation, was down-regulated in conjunctival epithelial cells of SS. Several reports have showed that expression of these genes is inhibited by IFN-gamma (Daireaux et al., 1990; Ramana et al., 2000; Ghosh et al., 2001). These reports and our results are fairly consistent with our hypothesis that IFN-gamma is a critical cytokine for the pathogenesis of SS though several other cytokines have up- or down-regulating effects on expression of these genes (Tiggesman et al., 1995).

Aquaporin 3 is a membrane water channel protein expressed in various tissues including conjunctiva and cornea. This gene is supposed to play a role in controlling water balance in tissue. In corneal epithelial cells of bullous keratopathy patients, this gene is up-regulated (Ljubimov et al., 2002). In this disease, cornea is edematous due to decreased function of corneal endothelial cells. Up-regulated aquaporin 3 may compensate the weakened endothelial function to alleviate painful epithelial bulla. The fact that dry eye is the opposite condition to this waterlogged situation prompts us to speculate that decreased expression of aquaporin 3 is physiological response to prevent intra-epithelial dehydration in SS. The fact that this gene is dominantly expressed in the epithelium of the bulbar conjunctiva than those of cornea (Hamann et al., 1998) also suggests an important role of this gene in controlling the water balance especially in conjunctival epithelial layer.

The remaining down-regulated genes, LOC92482, mesothelin, MDS033, and GS16785, GS16856, GS14618 (which presently have no definition) are mostly functionally unknown with reference to a possible pathological role in SS.

De Saint Jean et al. (1999) and Tsubota et al. (1999) proposed that IFN-gamma plays an important role in ocular surface inflammation in SS. Moreover, many of the genes that were up-regulated in SS, i.e. keratin 16, -17, MIG, amphiregulin, c-fos, fibronectin, and HLA-DR, have been shown to be affected by IFN-gamma in a variety of cell types. However, the up-regulation of ICAM-1 on the epithelial cells in SS has been reported (Jones et al., 1994); we were not able to ascertain this in our study.

The observation that IFN-gamma was up-regulated in the lacrimal gland (Saito et al., 1993) suggests that the elevation of tear IFN-gamma derives from infiltrating T lymphocytes in the lacrimal gland. Increased concentration of tear IFN-gamma could stimulate conjunctival epithelial cells to express various genes described above. However, a recent study demonstrated that activated T lymphocytes were seen in sub-conjunctival layer of not only SS patients but also non-SS dry eye patients whose lacrimal glands were not destroyed by activated T lymphocytes (Stern et al., 2002). These observations could imply that inflammation on the ocular surface of SS and non-SS dry eye patients is caused by common pathological events such as, increased shear stress over ocular surface due to decrease in lubrication or changes in sex-hormone supply. Also, T lymphocytes that infiltrate to substantia propria of conjunctiva were mainly composed of CD4 + and CD8 + T lymphocytes. These T lymphocytes could be sources for IFN-gamma, which induce conjunctival epithelial cells to express various genes in SS patients.

The similarity in gene expression patterns between SS and psoriasis is very interesting. In psoriatic skin, keratin 6, -16 and -17 (de Jong et al., 1991; Thewes et al., 1991) and several keratinization-related genes such as SPRR2A are up-regulated (Holh et al., 1995); these genes were also increased in conjunctival epithelial cells of SS. In addition, KLK7 (Ekholm and Egelrud, 1999), MIG (Goebeler et al., 1998), amphiregulin (Piepkorn, 1996), fibronectin (Bernard et al., 1985), ICAM-1 (Smith and Barker, 1995), and defensin beta 2 (Harder et al., 1997) are also up-regulated in both SS and psoriasis. Some psoriasis patients suffer from a relatively mild degree of dry eye in which they have a short tear film break-up time and squamous metaplasia in conjunctival cells evaluated by impression cytology (Karbubul et al., 1999). In this autoimmune disease, IFN-gamma is thought to be a critical cytokine (Wei et al., 1999) and we suggest that it plays a critical role in the pathogenesis of both SS and psoriasis as many of the genes up-regulated in SS are also induced by IFN-gamma. Though we did not examine conjunctival epithelial cells of
this disease, we suspect that systemic or local skin inflammation around eye may cause ocular surface inflammation through IFN-gamma or some other cytokines.

Considering our results and the results of others, we posit that abnormal differentiation and keratinization occur in the conjunctival epithelial cells of SS patients. We also propose that conjunctival epithelial thickening in SS may be attributable to the up-regulation of amphiregulin and/or c-fos and that the persistent punctate conjunctival erosions may not be due only to dryness but also to up-regulated KLK7 expression. We support the suggestion of De Saint Jean et al. (1999) and Tsubota et al. (1999) that IFN-gamma plays a critical role in the pathogenesis of the ocular surface inflammation presented by patients with SS.

However, although our results suggest that IFN-gamma has a role in the up-regulation of certain genes in SS, they do not permit broad conclusions regarding IFN-gamma induction of keratin 6b, -6c, -16, -17, fibronectin, amphiregulin, c-fos, and MIG in these cells. It could be argued that cytokines interact mutually and construct complicated organic networks and that many genes other than IFN-gamma may be involved in the pathogenesis of SS. Furthermore, Stern et al. (1998) describes many other possible causes, other than IFN-gamma, involved in the pathogenesis of SS, via activation of T lymphocytes, such as a decrease in androgen level or an interruption of the neural signal to lacrimal gland and conjunctiva.

At present we cannot rebut such objections. However, to our knowledge, this is the first study to demonstrate the up-regulation of fibronectin, MIG, defensin beta 2, keratin 6b, -6c, -16, KLK 7, SPRR2A, c-fos, and amphiregulin in conjunctival epithelial cells of SS patients. It is also the first to raise the possibility of a minimally invasive, gene expression-based diagnosis of SS.

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References


