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## Gene expression profiling in autoimmune non-infectious uveitis disease

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### Abstract

Non-infectious uveitis is a predominantly T cell mediated autoimmune, intraocular inflammatory disease. To characterize the gene expression profile from patients with non-infectious uveitis, peripheral blood mononuclear cells (PBMCs) were isolated from 50 patients with clinically characterized non-infectious uveitis syndrome. A pathway-specific cDNA microarray was used for gene expression profiling and real time PCR array for further confirmation. Sixty-seven inflammation and autoimmune associated genes were found differentially expressed in uveitis patients with twenty-eight of those genes being validated by real-time PCR. Several genes previously unknown for autoimmune uveitis, including IL-22, IL-19, IL-20 and IL-25/IL-17E, were found to be highly expressed among uveitis patients compared to the normal subjects with IL-22 expression highly variable among the patients. Furthermore, we show that IL-22 can affect primary human retinal pigment epithelial cells by decreasing total tissue resistance and inducing apoptosis possibly by decreasing phospho-Bad level. In addition, the microarray data identified a possible uveitis-associated gene expression pattern, showed distinct gene expression profiles in patients during periods of clinical activity and quiescence, and demonstrated similar expression patterns in related patients with similar clinical phenotypes. Our data provides the first evidence that a sub-set of IL-10 family genes are implicated in non-infectious uveitis and that IL-22 can affect human retinal pigment epithelial cells. The results may facilitate further understanding of the molecular mechanisms of autoimmune uveitis and other autoimmune originated inflammatory diseases.

### Keywords

human; autoimmunity; non-infectious uveitis; microarray; peripheral blood mononuclear cells

### Introduction

Intraocular inflammatory disease or uveitis is a major cause of visual handicap in the United States, causing an estimated >100,000 new cases of ocular morbidity per year and 10% of the new cases of legal blindness (1). It has been generally accepted that non-infectious uveitis is an autoimmune disorder predominantly mediated by T helper cells (2,3). Earlier studies on

humans as well as in animal models demonstrated that Th1 mediated autoimmune responses play a pivotal role in this disease (4–6). Several autoantigens have been identified as potential triggers for autoimmune uveitis (7–9). However, the varied clinical appearance in humans and the varied response to immunosuppressive regimens suggest that multiple mechanisms may be involved. For example, less than a third of uveitis patients tested will have T cell responses to arrestin, or S-antigen, the most recognized autoantigen for uveitis in humans (10). In addition, susceptibility to the two most important antigens used to induce experimental animal uveitis, interphotoreceptor retinoid-binding protein (IRBP) and S-antigen, varies from species to species despite evolutionarily conserved structures (11) and documented T cell response from uveitis patients to IRBP has been scarce. Recent work has also indicated that, similar to multiple sclerosis (MS), Th17 cells may be one of key immune components that contribute to the molecular pathogenesis of autoimmune uveitis (12). To facilitate the understanding of the molecular mechanisms of autoimmune uveitis and other autoimmune diseases, we investigated the gene expression profile of autoimmune uveitis using cDNA microarray and real time PCR array on 50 clinically characterized uveitis patients. Our results identified a set of IL-10 family genes, including IL-22 that are highly expressed in uveitis patients when compared to that of normal subjects, but have not been previously implicated in non-infectious uveitis. An in vitro study demonstrated that IL-22 affected human primary retinal pigment epithelial cells by decreasing their total tissue resistance, a hall mark of tissue integrity of those retinal epithelial cells (13). Meantime, IL-22 induced apoptosis of cultured human primary retinal pigment epithelial cells which is associated with decreased phospho-Bad level. We also show that there exists a molecular pattern for gene expression among uveitis patients compared to normal controls.

## Materials and Methods

### Patients, normal controls and human fetal tissue

The research performed in this study followed the tenets of the Declaration of Helsinki and the NIH Institutional Review Board. Fifty-eight patients with well defined clinical diagnoses of non-infectious uveitic syndrome were enrolled into the National Eye Institute (NEI) after IRB approval (protocol #02-EI-0122). Patient consent was obtained prior to enrollment and the ocular status of the patients after enrollment was evaluated and recorded independently by ophthalmologists in the NEI Uveitis Clinic. Samples from 20 healthy donors as normal controls were obtained from the NIH Blood Bank (protocol #97-0134). Human fetal eyes of nominal gestation of 15 to 17 weeks were obtained from Advanced Bioscience Resources (Alameda, CA).

### Cell culture on transwell filters

Primary cell cultures of human fetal retinal pigment epithelial (hFRPE) cells were prepared from human fetal eyes as described previously (13). Second passage cells were seeded in transwells (Corning Costar, 0.4 $\mu$ m pores, polyester membranes). Media were changed every three days and the cultures were maintained for at least 3 weeks before experiments.

### cDNA microarray analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from patients or normal donors as described previously (14). Total RNA was extracted using an RNeasy kit (Qiagen, CA). RNA isolated from 20 healthy donors were pooled and served as normal control. Biotinylated probes were generated from 5  $\mu$ g of total RNA from each RNA sample by incorporating biotinylated dUTP into synthesized cDNAs using a T7-polymerase linear amplification strategy. Probes were hybridized to a pathway-specific cDNA microarray chip (Inflammatory and autoimmune GEArray; SuperArray Inc., Gaithersburg, MD). Signals for specific binding were recorded by a CCD camera. A total of 51 microarray analyses were performed independently for all patient

samples and one pooled normal sample. Gene expression profiling was analyzed using GEArray Suite software (SuperArray Inc., MD). Briefly, acquired digital images were aligned, computed for density, normalized based on negative controls and transformed into scores for gene expression based on an established algorithm (SuperArray Inc., MD). All scores for each gene in one array were then normalized on house-keeping genes, e.g. GAPDH and beta-2-microglobulin (B2M). The final scores of each of the genes of all arrays were then compared to those from a control array to obtain a ratio value. A 2-fold cut-off threshold was used to define if one gene is up-regulated or down-regulated, e.g., the expression of one gene from array X is equal or greater than 2-fold of that from control array is considered up-regulated gene while equal or less than 0.5-fold of the score from the same gene compared to the control array is considered down-regulated gene. The scatter plot and clustergram were acquired using the same software according to the manufacturer's instructions.

### Real-time PCR analysis

Real-time PCR array analysis was performed to confirm microarray results. A total of 60 autoimmune and inflammatory disease related genes were examined for confirmation purposes by real-time PCR using a commercially available real-time PCR RT<sup>2</sup> Profiler kit (SuperArray, MD). To correlate the clinical status of the uveitic condition to the gene expression profile, only samples from patients with clinically active disease were used for real-time PCR analysis. Active uveitis was defined as evidence of cells and flare in the anterior chamber of the eye or cells and haze in the posterior chamber of the eye. A total of 42 RNA samples from clinically active uveitis patients were pooled to compare to the pooled normal control sample. Two independent PCR array analyses were performed according to the manufacturer's instructions. Briefly, 1 µg of each of the RNA samples from either uveitis patients or normal controls were reverse transcribed into cDNAs using a first strand cDNA RT kit (SuperArray, MD). Real-time PCR was performed using a 96-well format PCR array and an ABI 7500 real-time PCR unit (ABI, CA). Primers for all genes for real-time PCR confirmation of the microarray analysis had been pre-tested and confirmed by the manufacturer. Assay controls include positive and negative controls as well as 3 sets of house-keeping gene controls for normalization purposes. Analysis of real-time PCR results was performed following the manufacturer's instructions (SuperArray Inc., MD). Data analysis is based on the  $\Delta\Delta C_t$  method with normalization of the raw data to housekeeping genes as described in the manufacturer's manual. The detailed instruction for calculation and normalization can be found in the following website: [www.superarray.com](http://www.superarray.com). A 2-fold cut-off threshold was used to define if one gene is up-regulated or down-regulated. To further confirm IL-22 expression results based on microarray and real-time PCR array data, a SYBR Green based qRT-PCR was used to analyze IL-22 expression between normal and uveitis patients. Briefly, RNA samples from 6 normal donors and 40 uveitis patients were tested for IL-22 gene expression using the qRT-PCR MasterMix from SuperArray Inc. following their Endpoint RT-PCR Manual (Gaithersburg, MD). Human GAPDH gene expression from the same RNA samples was also tested for normalization and quantification purposes. The results were expressed as the fold expression of IL-22 normalized on that of GAPDH. The IL-22 expression was considered not detectable if the ratio of IL-22 against GAPDH is smaller than 0.001. qRT-PCR primers for IL-22 and GAPDH were both from SuperArray Inc. and have been verified by the provider.

### Western blot analysis

fRPE cells were cultured in the presence or absence of 50ng/ml of recombinant human IL-22 (PeproTech, Rocky Hill, NJ) for 48 hours. Cells were then lysed in 100 µl of lysis buffer [50 mM Tris-Cl, 1% Triton X-100, 100 mM NaCl, 2 mM EDTA, 50 mM NaF, 50 mM glycerol-phosphate, 1 mM NaVO<sub>4</sub> and 1X protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. Complete cell lysis was achieved by immediately vortexing and then boiling in an equal amount of 2 X SDS protein loading buffer at 95°C for 5 minutes. Cell debris was

removed by centrifugation at 12,000 rpm for 3 min. Twenty microliter of each sample was loaded into a 12% SDS-polyacrylamide gel with a 4% stacking gel. For immunoblotting, primary antibody of anti-phospho-Bad was purchased from Cell Signaling Technology (Beverly, MA). Anti- $\beta$ -actin antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

### Resistance and apoptosis assay for RPE cells

The RPE monolayers on inserts with total tissue resistance (TER) above  $100 \Omega\text{-cm}^2$  were cultured with either RPE culturing media alone or in the presence of 50 ng/ml of human recombinant IL-22 (PeproTech, Rocky Hill, NJ). After 72 hours of stimulation with IL-22 of RPE monolayers on inserts, TER was measured using EVOM (WPI, FL). Data are expressed as mean  $\pm$  S.E.; statistical significance (Student's t test, two-tail) was accepted as  $p < 0.05$ . For apoptosis analysis, experimental cells from the same experiments as described above were stained by Annexin (BD Bioscience, CA) after 72 hours culturing. Samples were acquired by a FACSCalibre flow cytometer and analyzed using FlowJo software (TreeStar, San Jose, CA).

## Results

### Differential gene expression among uveitis patients and normal subjects

A total of 58 patients were enrolled into our microarray protocol. Table 1 summarizes the clinical information of all patients who were enrolled. The diagnoses, gender, race and clinical manifestations of the enrolled patients represent what is typically seen in the Uveitis Clinic of the National Eye Institute. Eight RNA samples did not qualify for the microarray analysis after quality control test. To test the validity of our strategy to use pooled RNA samples from normal donors as reference RNA sample to compare to individual RNA samples from uveitis patients, we ran microarray on 9 RNA samples, 8 individual RNA samples from normal donors vs. the pooled RNA sample from the same donors. A side-by-side analysis showed that the data derived from the 8 individual donors vs. the pooled sample from normal donors are very similar. Based on statistical correlation analysis, the average p value of correlation of the microarray data between the pooled vs. the 8 individual RNA samples is 0.88 (0.86–0.91). In addition, Clustergram analysis also demonstrated that there was a great similarity of gene expression patterns among the 8 individual normal donors when compared to the pooled RNA sample from the same donors (data not shown). Therefore, a total of 51 RNA samples, 50 from patients and one reference RNA pooled from 20 healthy donors, were analyzed for microarray analysis. Despite the high heterogeneity of gene expression among patients and between patients and controls, there were clear differences in gene expression patterns comparing those from patients to those from the normal pooled control RNA. Figure 1 is a scatter plot analysis showing genes that were differentially expressed among uveitis patients when compared to the normal donors with red symbols representing up-regulated genes and green symbols representing down-regulated genes. When a 2-fold cut-off threshold was applied, there were a total of 67 genes (16.7%) that were differentially expressed among uveitis patients when compared to normal controls with 56 genes up-regulated and 11 genes down regulated among the 400 inflammatory and autoimmune diseases associated genes in this pathway-specific cDNA array chip. A complete list of differentially expressed genes (2-fold cut-off) from cDNA microarray results for uveitis is provided in Table 2.

### Gene expression profiling revealed 4 distinct sub-groups of uveitis patients

Non-infectious uveitis is considered a heterogeneous group of diseases comprised of a variety of clinical diagnoses and manifestations but all demonstrating intraocular inflammation. Based on this notion, we compared the differential gene expression patterns among uveitis patients using a density clustergram analysis with a red colored band representing higher and green representing lower gene expression after normalization to controls. There were clearly four

distinct profiles (I, II, III and IV) based on their unique patterns of gene expression (Fig. 2). A detailed analysis of genes in those four profiles revealed 4 sets of genes with higher expression that define the differential gene expression profiling. A gene list for those 4 sets of genes, termed as signatures A, B, C and D is shown in Fig. 3. It is of interest that the genes within each set appeared functionally related. For example, genes in set A were more functionally associated with cell proliferative responses, genes in set B were more associated with cell apoptosis while most cytokine/chemokines were clustered in the set C and set D. One intriguing question is whether these molecular signatures reflect distinct molecular pathogenesis for autoimmune uveitis. However, our efforts to correlate clinical uveitic diagnoses with this differential gene expression profiling were not successful (see Discussion for details).

### **Real-time PCR validated gene list provides evidence of involvement of previously uncharacterized genes in autoimmune uveitis**

We then used quantitative real-time PCR to validate the microarray results. As shown in Table 3, real-time PCR analysis confirmed 18 up-regulated genes and 10 down-regulated genes in active uveitis patients as compared to those in normal controls (2-fold cut-off threshold). Some of the genes have been previously reported in association with autoimmune uveitis, e.g. CX3CR1, CCL19, CCL21, FasL, IFN- $\alpha$ , IL-4, -5, -6 and IL-8. However, several genes identified have not been previously associated with autoimmune uveitis including several cytokines from the IL-10 family and one cytokine from the IL-17 family. One set of IL-10 family genes including IL-19, IL-20 and IL-22 were of particular interest. IL-10 has been implicated to play a protective role in the mouse EAU model and is up regulated in human uveitis patients (6,15,16). However, IL-19, IL-20 and IL-22 have not been implicated in human uveitis. In addition, IL-25/IL-17E, which belongs to the IL-17 cytokine family, has also not been reported to be associated with autoimmune uveitis. Genes that have not been previously reported to be associated with autoimmune uveitis are highlighted in bold and italic in Table 3. To further confirm the microarray and real-time PCR array data, we tested IL-22 gene expression in 6 normal donors and 40 uveitis patients by using a SYBR Green based qRT-PCR strategy. As shown in Figure 4, five out of six normal donors had no detectable IL-22 transcript except 1 donor showed very low level of IL-22 expression by real-time PCR. However, 25 out of 40 (62.5%) uveitis patients tested positive for IL-22 expression with some patients demonstrated very high levels of IL-22 expression.

### **IL-22 affects human primary pigment epithelial cells by decreasing their total tissue resistance and inducing apoptosis**

The expression of IL-22 has been recently shown to be associated with Th17 cells (17,18). The primary target cells for IL-22 appeared to be epithelial cells(19,20). To further understand the possible physiological impact and molecular mechanisms of IL-22 on ocular disease, we examined the effect of IL-22 on total epithelial resistance (TER) and the induction of apoptosis of human retinal pigment epithelial (RPE) cells. The retinal pigment epithelium forms the outer blood-retina barrier which physically separates the subretinal space (SRS) and the choroidal blood supply and actively participates in the creation of the immune privileged retinal environment by secreting various cytokines and chemokines (unpublished data). Both TER and apoptosis assays examine the physiological integrity of the retinal pigment epithelium, which is critical to preservation of the outer blood-retina barrier. As shown in Fig. 5, after 72 hours of stimulation, IL-22 significantly decreased total epithelium resistance (TER) by more than 60% as compared to matched controls ( $p < 0.01$ ). TER decreased from  $742 \pm 29$  to  $296 \pm 37$   $\Omega \cdot \text{cm}^2$  (Panel A). IL-22 also induced RPE apoptosis when compared to the controls (Panel B). To further understand the mechanisms of IL-22 inducing apoptosis of RPE cells, we examined several proteins that are known to be involved in apoptosis signaling pathway, including cleaved caspase 3 and PARP, and phospho-Bad. We found that IL-22 seemed primarily targeting phosphorylated-Bad. As shown in Figure 5C, IL-22 treatment resulted in decreased

phospho-Bad expression in RPE cells (panel C). However, there was no significant change in the levels of cleaved (active) caspase 3 and PARP expression which are also known to be involved in the apoptosis pathway (data not shown). This is the first evidence that IL-22 can target the human retinal pigment epithelial cells and potentially change their tissue integrity and preservation of the blood retinal barrier.

### Gene expression profiling on patient samples at distinct clinical stages

An important characteristic of uveitis is recurrence of disease (21). However, it is not clear for clinicians if this is a new inflammatory episode or is simply still the previous episode that has lingered. To address this question, we continuously monitored one patient over a 5-month span and collected samples during 3 distinct periods of disease activity, e.g. active, quiescent and recurrent/active stages. Interestingly, there were very few changes in gene expression profiling for the samples collected at the three time-points which represented three distinct clinical stages. As shown in Figure 6, arrays 1, 2 and 3 represent gene expression patterns when the disease was at clinically active, quiescent and recurrent/active stages respectively. The expression profiles (red=higher expression, green=lower expression after normalized with normal control group) of the 3 arrays were strikingly similar, notice that the colors distribution of the three profiles are very similar, suggesting the continuity of the underlying disease despite temporary relieve of clinical symptoms. However, analyses and data from more patients with similar clinical manifestations are required to further validate this initial observation.

### Gene expression profiling on samples from siblings with the same clinical diagnoses

Several lines of evidence suggest that genetic factors play an important role in autoimmune uveitis (22–24). During our study, we were able to enroll two male siblings, both diagnosed with intermediate uveitis. We performed a microarray analyses on their blood samples. Shown in Figure 7 is a clustergram comparing the gene profiling results of the siblings (labeled as patient A and patient B) vs. the pooled normal control samples (labeled as NL). The gene profiling patterns of the siblings (patient A and patient B) were dramatically different from that of normal controls, highlighting molecular evidence of differential gene expression of non-infectious uveitis patients. However, the expression profiles between the two siblings are very similar to each other (compare patient A to patient B). This is the first microarray analysis on such uveitis patients and the data further confirms the notion that genetic factors play an important role in ocular autoimmune diseases.

## Discussion

The rationale to use pathway-specific cDNA microarray representing a portion of human genome is not only due to cost/efficiency consideration but is more importantly based on the fact that accumulated data have demonstrated that only a fraction of genes are actively transcribed (25). In addition, previous studies have provided strong evidence that non-infectious uveitis is an autoimmune, intraocular inflammatory disease (21). This study was initiated to investigate molecular profiles of non-infectious uveitis at the gene expression level. Therefore, using a pathway-specific cDNA microarray designed for autoimmune and inflammatory diseases is one of the reasonable strategies to meet our goals. We have analyzed RNA samples from 50 non-infectious uveitis patients using an inflammatory and autoimmune focused cDNA microarray. About 17% of the genes in this focused array were either up- or down-regulated in the uveitis patients when compared to normal controls. To the best of our knowledge, this is the first pathway specific and genome-wide demonstration of differential gene expression between non-infectious uveitis patients and normal controls. Since the RNA samples were purified from fresh PBMCs isolated immediately from whole blood, we believe that this differential gene expression pattern likely reflects the differential gene expression due to the disease activity of uveitis. By using clustergram analysis, we are able to classify uveitis

into 4 sub-groups based on gene expression profiling results (Fig. 2). In addition, we have identified 4 sets of genes which define the four sub-groups of uveitis patients, designated as molecular signatures A, B, C and D. Although not all genes within each set were able to be completely categorized into a single general function, the genes within each set appear to be functionally related (Fig. 3).

The primary purpose for gene-expression profiling on autoimmune uveitis is to further understand molecular mechanisms and discover new pathways for this important ocular disease. Based on cDNA microarray data and real-time PCR validation data, we identified 28 genes that were either up- or down-regulated in the peripheral blood of uveitis patients with active disease when compared to normal control (Table 3). The majority of those genes (19 out of 28) have been previously reported to be associated with autoimmune uveitis, further validating the results of our study. However, among those up-regulated genes confirmed by real-time PCR, a set of IL-10 family genes, including IL-19, IL-20 and IL-22 which have not been previously associated with autoimmune uveitis are of particular interest. IL-10 has been implicated to play a protective role in the mouse EAU model and is up regulated in human uveitis patients (6,15,16). However, IL-19, IL-20 and IL-22 have not been implicated in human uveitis. The expression of IL-22 has been recently associated with Th17 cells (17,18), a newly characterized T helper cell sub-population that are believed to primarily contribute to the pathogenesis of some Th1 mediated autoimmune diseases such as multiple sclerosis(26–28). Th17 cells have also been implicated in the pathogenesis in a mouse uveitis model(12). IL-22 has little regulatory effect on immune cells but has primarily an effect on target tissues (29). It has been suggested that IL-22 may play an important role in autoimmune diseases such as psoriasis and ulcerative colitis (19,20). Recently, IL-22 has been shown to interrupt the blood-brain barrier (BBB) tight junctions both in vitro and in vivo and it therefore promotes Th17 cells to transmigrate through the blood-brain barrier (30). The blood-retina barrier, similar to the blood-brain barrier, is well-known to be critical for the maintenance of intraocular homeostasis. Since Th17 cells have been implicated in the pathogenesis of autoimmune uveitis (12), we tested the effect of IL-22 on human primary retinal pigment epithelium (RPE). The data shows that IL-22 dramatically reduces total tissue resistance of human RPE cells and that IL-22 also induces RPE apoptosis (Fig. 5). The decrease of total epithelium resistance (TER) and the induction of apoptosis of RPE cells by IL-22 may have significant physiological and pathophysiological consequences in the eye. Epithelial electrical resistance measurement can be directly correlated to some barrier properties where in many cases TER decrease would result in an epithelial monolayer permeability increase (31–33). The reduction in TER would be consistent with the opening of a pathway that would allow biologically active molecules and immune system components to reach the site of retinal inflammatory injury in vivo(34, 35). Our data also showed that IL-22 decreased phospho-Bad level (Fig. 5C) and that might contributed to the induced apoptosis of fRPE cells. This data has not been reported in the literature and further investigation on the effect of IL-22 on phospho-Bad and its downstream signaling pathway will be very interesting. Overall, the data of IL-22's effect on total tissue resistance as well as on inducing apoptosis suggests that IL-22 can affect the tissue integrity of RPE cells. Since RPE cells are critical for maintaining the blood-retina barrier, we would propose that IL-22 may target human RPE cells. Similar to what have been demonstrated in the multiple sclerosis that IL-22 affects on blood-brain barrier (30), IL-22 may disrupt the blood-retina barrier by reducing RPE total epithelial resistance and inducing RPE apoptosis. We suggest that this may be one of molecular mechanisms for uveitis pathogenesis. Our findings provide the first evidence that directly links IL-22 with human intraocular inflammatory disease.

One of the other applications of gene-expression profiling for clinical immunology is for molecular diagnoses such as in cancer (25,36) or in autoimmune and inflammatory diseases (37,38). We sought to correlate those four sub-groups of gene-expression profiles with clinical

diagnoses and manifestations. However, we were not able to directly link the gene expression profiling data with clinical diagnoses. For example, patients with the diagnosis of intermediate uveitis can exhibit all four of the gene expression profiles. It is an intriguing question as to whether these molecular signatures can reflect distinctive molecular pathogenesis for autoimmune uveitis. Since all patients received at least 2 immunosuppressive agents for therapy, it is unlikely that immunosuppression would cause significant impact on the distinct molecular profiling results which resulted in the failure of correlation of clinical manifestation with the molecular profiling data. We propose, based on our data, that clinical manifestations may not necessarily be able to be correlated with molecular pathways of uveitic syndromes. In fact, similar observations have been made in large-B-cell lymphoma with no common set of genes expressed in all cases (39–41). We suggest that our data provided further evidence that underscore current challenges in the reconciliation of molecular signatures vs. clinical diagnoses. However, we can not completely exclude the possibility that this can also be simply due to the fact that there were not enough samples to reveal all possible patterns. Although we performed 51 microarray (50 patients plus 1 pooled normal control) analyses for this study, using this particular cDNA chip, a relatively small number of patients were represented in each sub-group of uveitis (table 1).

Nevertheless, the data for the first time demonstrate a unique pattern of differential gene expression between autoimmune uveitis patients and normal donors, as well as possible molecular sub-types among autoimmune uveitis patients. To further explore the applications of gene-expression profiling in autoimmune uveitis, we examined two other clinically relevant issues. The first question is if there is a difference of gene expression pattern for the same patient at different clinical stages. Many uveitis patients will typically experience multiple episodes of occurrence during the course of their disease. However, it is not well defined if those episodes were new or merely a continuation of the previous one. We examined the gene expression profiles of one particular patient with three defined clinical stages, active, quiescent and recurrent/active over a span of more than 5 months. We were surprised to see that the gene expression profiles from the three distinct stages are overall very similar (Fig. 6). This is a clear demonstration that the underlying mechanisms, represented by the consistent gene-expression profile, have not been changed despite temporary clinical regression with therapy. We propose that this represents strong molecular evidence that clinical quiescence may not be a true reflection of underlying disease inactivity for autoimmune uveitis. Molecular analyses, such as gene profiling, may prove to be a more reliable and accurate method in predicting the clinical prognosis for recurrent diseases. We also examined gene-expression profiles of a pair of male siblings who both have intermediate uveitis as a diagnosis. The gene expression profiles of the siblings were very similar to each other but dramatically different from the profile of normal controls (Fig. 7). Consistent with previous studies in both human and in a mouse uveitis model (22–24), our initial study on gene-expression profile data from 2 male siblings who suffered from the same uveitis support the hypothesis that genetic factors are important in the pathogenesis of human autoimmune uveitis. However, more data will be needed to further validate this initial observation.

This study not only implicated IL-22 in human uveitis, but also the involvement of both IL-19, IL20 and IL-25/IL-17E. It is of interest that IL-19 and IL-20, similar to IL-22, all belong to the IL-10 family. They share many biological characteristics with IL-22 and are shown to be associated with inflammatory disease (29). IL-25/IL-17E has been shown to boost type 2 immune response and allergy (42,43). Although we do not currently understand the molecular mechanisms of the contribution of those cytokines to autoimmune uveitis, our finding that all those three genes are up regulated in human autoimmune intraocular inflammatory disease may suggest a novel mechanism that contributes to human autoimmune intraocular inflammatory disease (uveitis). Consistent with a recent report that Th17 cells are involved in the pathogenesis of autoimmune uveitis(12), the finding that IL-22, IL-19, IL-20 and IL-25/



IL-17E may contribute to the pathogenesis of autoimmune intraocular inflammatory disease sheds new insight on the molecular mechanisms of uveitis, as well as other autoimmune inflammatory diseases.

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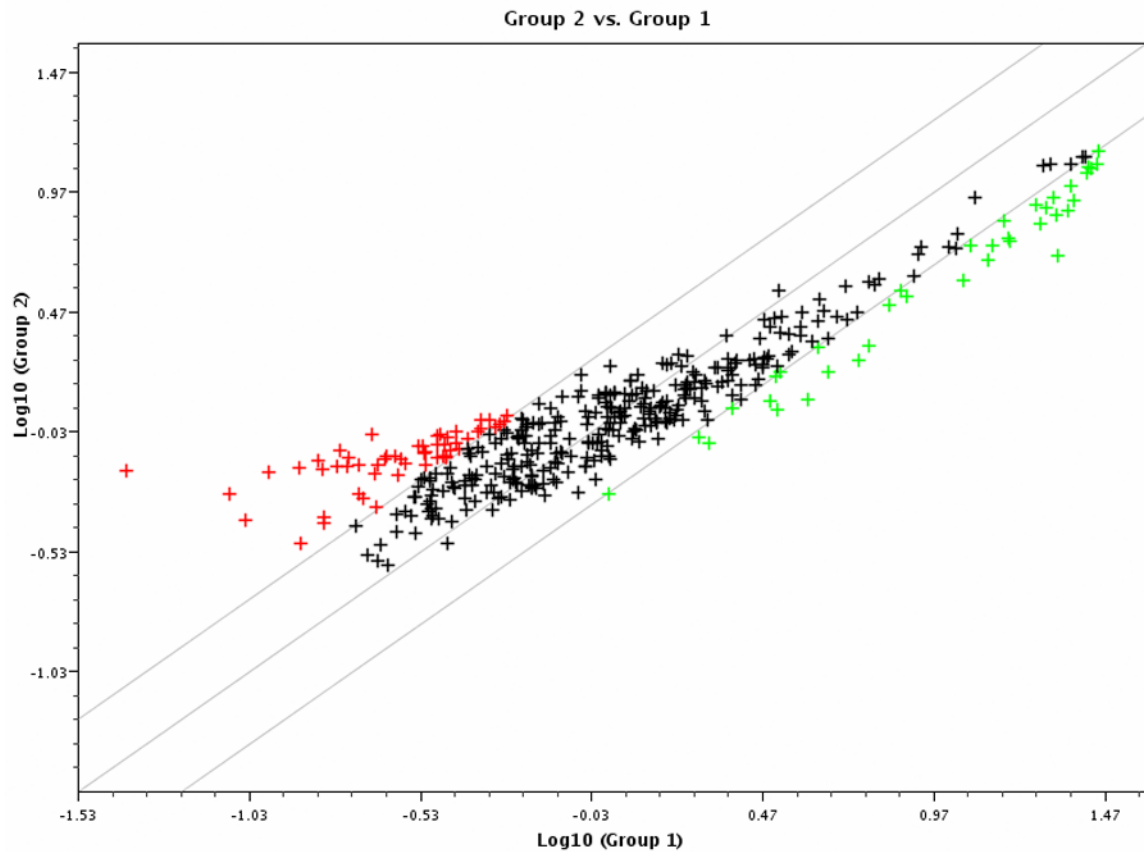
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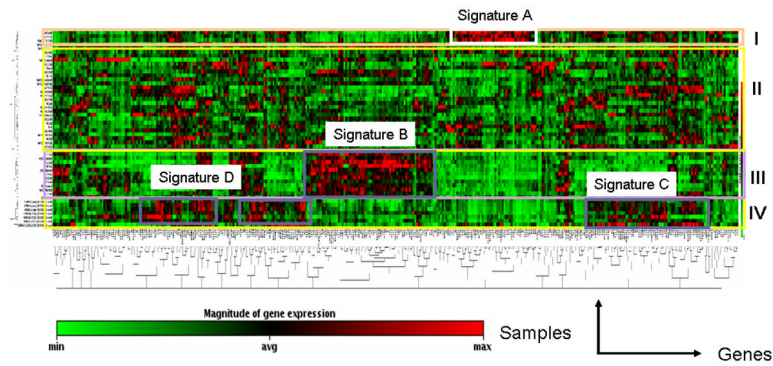
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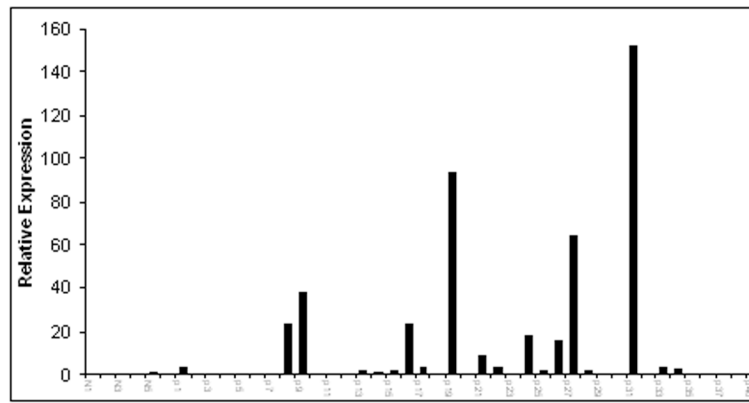
**Figure 1.**

Scatter plot analysis of gene expression profiling on uveitis patients demonstrates differential gene expression. cDNA microarray analysis on 50 RNA samples from uveitis patients and 1 pooled sample from controls was performed. After normalization on house-keeping genes, e.g. GAPDH and beta-2-microglobulin (B2M), the final scores of each of the genes of all arrays were compared to those from the control array. The scatter plot was acquired as described in the Materials and Methods. The Y-axis represents log scores from uveitis patients (group 2) and the X-axis represents log scores from normal controls (group 1). Each symbol represents one gene with red-colored ones represent 2-fold higher expressed in uveitis patients (group 2), green-colored ones represent 2-fold lower expressed in uveitis patients and the black-colored ones represent within 2-fold cut-off threshold.



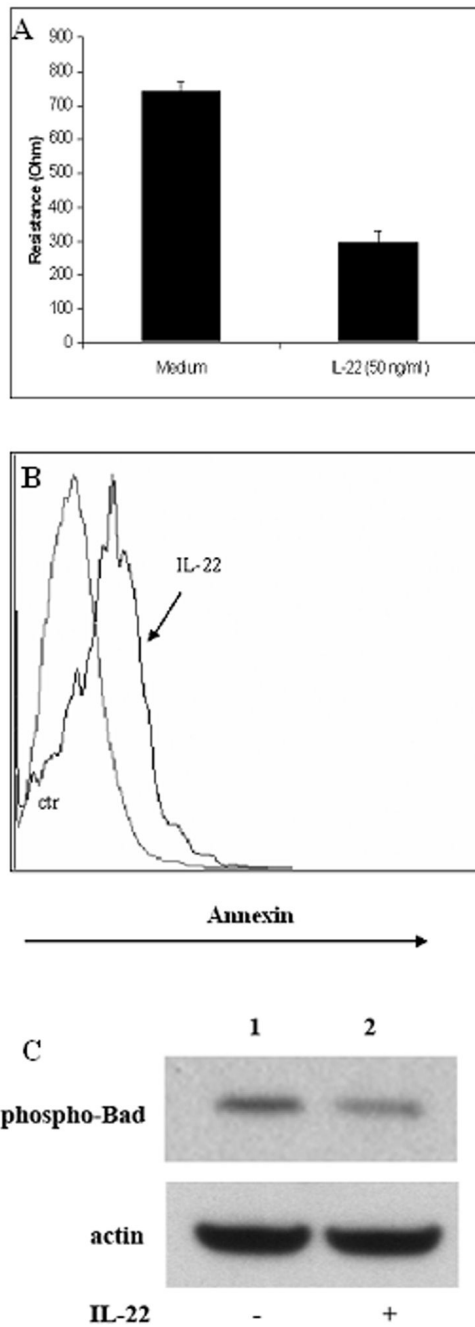
**Figure 2.** Clustergram analysis of gene expression revealed 4 distinct gene expression profiles. cDNA microarray was performed as described above and clustergram analysis on microarray data was described in the Materials & Methods section. X-axis represents all genes listed in the cDNA microarray chip and Y-axis depicts gene expression profiles from all samples of uveitis patients normalized on normal controls. Each colored band represents expression of a single gene from one patient when compared to that from normal donors with higher expression in red and lower expression in green. Groups I, II, III and IV designate 4 sub-groups of gene expression profiles. Analysis of gene expression profiling among uveitis patients revealed 4 sets of genes designated as molecular signatures A, B, C and D that can be used to define the 4 sub-groups of gene expression profiles.





**Figure 4.**

The expression of IL-22 in normal donors and uveitis patients. A SYBR Green based Real-time PCR analysis was performed to analyze IL-22 mRNA levels in both normal donors and uveitis patients as described in the Materials and Methods. Each bar on the X-axis represents one RNA sample from either normal donors or uveitis patients subjected to real-time PCR analysis for IL-22 transcript. The Y-axis represents relative expression of IL-22 levels.

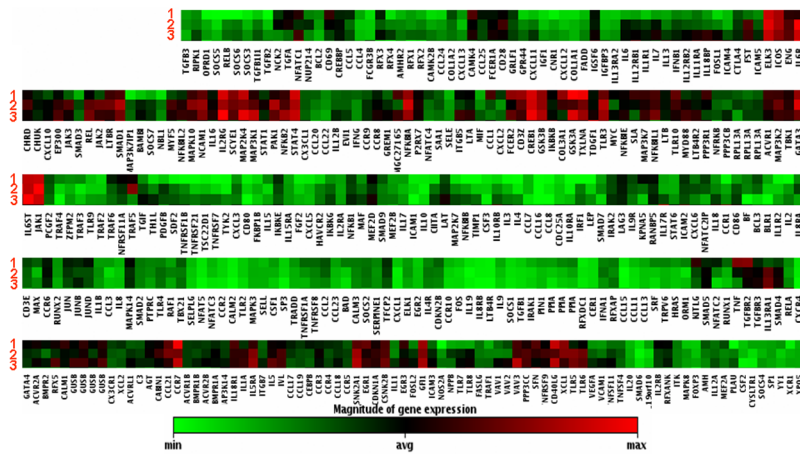


**Figure 5.**

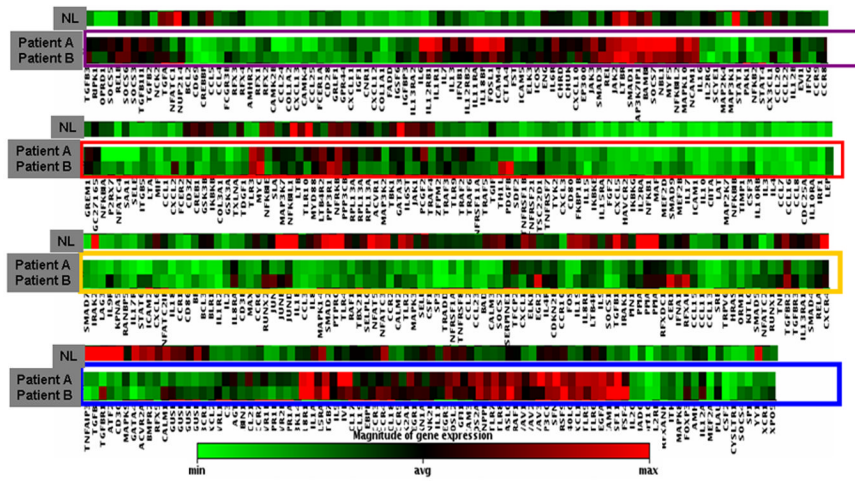
IL-22 decreased total tissue resistance and induced apoptosis of primary human retinal pigment epithelial (RPE) cells. Monolayers of RPE cells grown on inserts were treated with recombinant human IL-22 (50 ng/ml). Resistance measurements and an apoptosis assay were performed after 72 hours of incubation with IL-22 as described in the Materials & Methods. Panel A shows summary bar-graph of total epithelium resistance (TER) changes after addition of IL-22 to hfRPE monolayers. Compared to matched controls TER significantly (~ 60%) decreased in IL-22 treated monolayers of RPE (n=3, p<0.01). Panel B is a histogram comparing Annexin staining for human primary RPE cells with and without IL-22 treatment. The grey-colored line is for control group while the bolded dark line for IL-22 treated RPE cells. There is a right shift



of Annexin staining for IL-22 treated RPE cells when compared to the non-treated control group, indicating increased apoptosis induced by IL-22 treatment. In panel C, monolayers of RPE cells grown on inserts were treated with or without recombinant human IL-22 (50 ng/ml) for 48 hours, cells were lysed and subjected to Western analysis for phosphorylated-Bad. Although the beta-actin levels were comparable comparing IL-22 treated and untreated groups, the phosphorylated-Bad was decreased after IL-22 treatment.



**Figure 6.** Clustergram analysis of gene expression profiles from samples for one patient collected at 3 clinically distinct stages. The numeric numbers represent different stages of the disease when the samples were collected and analyzed. Stage 1 is when the patient was having an initial active ocular inflammatory episode; stage 2 was when the disease quiescent and stage 3 when there was another active/recurrent episode. The X-axis listed all the gene symbols. Each colored band represents expression of a single gene from the patient at a specified clinical stage when compared to that from normal donors with higher expression in red and lower expression in green. Notice that the overall gene expression profiling of the samples at three stages is very similar.



**Figure 7.** Comparison of clustergram analysis of gene expression profiling of 2 male siblings (designated as patient A and patient B) with the same diagnosis of intermediate uveitis with that from normal controls (designated as NL). The cDNA microarray and clustergram analysis were performed as described above and in the Materials and Methods. The X-axis listed all the gene symbols in the cDNA microarray chips. The gene expression profiles from the 2 male siblings with the same diagnosis of intermediate uveitis (designated as patient A and patient B) were aligned with the profile from normal subjects (NL). As described above, each colored band represents expression of a single gene from either patient A or patient B or from normal subjects after normalization on house keeping genes, with higher expression in red and lower expression in green.

Table 1

## Patient information

	Asian Pacific	Black, not Hispanic	Hispanic	White, not Hispanic	Others	Total
Female	1	11	2	18	0	32(55%)
Male	2	5	1	17	1	26(45%)
Total	3	16	3	35	1	58
Mean Age=36.7 yrs. (Range 6-78 yrs) <b>Enrollment</b> 13 7 6 5 5 4 4 3 2 13						
<b>Clinical Diagnosis</b>						
Intermediate Uveitis						
Behcet's						
Panuveitis						
Sarcoidosis						
VKH						
Anterior Uveitis						
Retinal vasculitis						
Scleritis*						
Others						

\* Include: multifocal choroiditis, serpiginous, etc.

**Table 2**  
Gene list of differentially expressed genes in uveitis patients

Position	UniGene	RefSeq No	Symbol	Description	Fold changes
48	Hs.10458	NM_004590	CCL16	Chemokine (C-C motif) ligand 16	16.6
171	Hs.168132	NM_172175	IL15	Interleukin 15	7.6
62	Hs.251526	NM_006273	CCL7	Chemokine (C-C motif) ligand 7	5.9
112	Hs.201300	NM_006639	CYSLTR1	Cysteinyl leukotriene receptor 1	5.6
174	Hs.41724	NM_002190	IL17	Interleukin 17 (cytotoxic T-lymphocyte-associated serine esterase 8)	5.0
192	Hs.73917	NM_000589	IL4	Interleukin 4	5.0
63	Hs.271387	NM_005623	CCL8	Chemokine (C-C motif) ligand 8	4.6
172	Hs.524117	NM_002189	IL15RA	Interleukin 15 receptor, alpha	4.4
64	Hs.301921	NM_001295	CCR1	Chemokine (C-C motif) receptor 1	4.4
80	Hs.1634	NM_001789	CDC25A	Cell division cycle 25A	4.1
222	Hs.498997	NM_014387	LAT	Linker for activation of T cells	3.8
252	Hs.153629	NM_005919	MEF2B	MADS box transcription enhancer factor 2, polypeptide B (myocyte enhancer factor 2B)	3.4
96	Hs.2233	NM_000759	CSF3	Colony stimulating factor 3 (granulocyte)	3.4
239	Hs.531754	NM_145185	MAP2K7	Mitogen-activated protein kinase kinase 7	3.3
185	Hs.272373	NM_018724	IL20	Interleukin 20	3.0
368	Hs.75516	NM_003331	TYK2	Tyrosine kinase 2	2.9
237	Hs.134859	NM_005360	MAF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	2.8
89	Hs.172928	NM_000088	COL1A1	Collagen, type I, alpha 1	2.8
157	Hs.43505	NM_003639	IKBKKG	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase gamma	2.7
254	Hs.497723	AF067420	MGC27165	Hypothetical protein MGC27165	2.7
158	Hs.193717	NM_000572	IL10	Interleukin 10	2.7
255	Hs.126714	NM_000246	CHITA	Class II, major histocompatibility complex, transactivator	2.7
127	Hs.513470	NM_032815	NFATC2IP	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein	2.7
253	Hs.314327	NM_005920	MEF2D	MADS box transcription enhancer factor 2, polypeptide D (myocyte enhancer factor 2D)	2.6
139	Hs.466828	NM_019884	GSK3A	Glycogen synthase kinase 3 alpha	2.6
335	Hs.517148	NM_016397	THIL	TH1-like (Drosophila)	2.5
191	Hs.694	NM_000588	IL3	Interleukin 3 (colony-stimulating factor, multiple)	2.5
160	Hs.418291	NM_000628	IL10RB	Interleukin 10 receptor, beta	2.5
236	Hs.123119	NM_005905	SMAD9	SMAD, mothers against DPP homolog 9 (Drosophila)	2.5
187	Hs.465645	NM_019107	C19orf10	Chromosome 19 open reading frame 10	2.4
169	Hs.336046	NM_000640	IL13RA2	Interleukin 13 receptor, alpha 2	2.4
328	Hs.133379	NM_003238	TGFB2	Transforming growth factor, beta 2	2.4
223	Hs.194236	NM_000230	LEP	Leptin (obesity homolog, mouse)	2.4
141	Hs.155111	NM_032782	HAVCR2	Hepatitis A virus cellular receptor 2	2.4
313	Hs.468426	NM_144949	SOC55	Suppressor of cytokine signaling 5	2.4
207	Hs.436061	NM_002198	IRF1	Interferon regulatory factor 1	2.3
170	Hs.17987	L15344	TXLNA	Taxilin alpha	2.3
7	Hs.465709	NM_000635	RFX2	Regulatory factor X, 2 (influences HLA class II expression)	2.3
121	Hs.86131	NM_003824	FADD	Fas (TNFRSF6)-associated via death domain	2.3
334	Hs.373550	NM_003244	TGIF	TGFB-induced factor (TAFE family homeobox)	2.3
67	Hs.184926	NM_005508	CCR4	Chemokine (C-C motif) receptor 4	2.3
6	Hs.73677	NM_002918	RFX1	Regulatory factor X, 1 (influences HLA class II expression)	2.3
256	Hs.407995	NM_002415	MIF	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	2.2
159	Hs.504035	NM_001558	IL10RA	Interleukin 10 receptor, alpha	2.2
109	Hs.89714	NM_002994	CXCL5	Chemokine (C-X-C motif) ligand 5	2.2
224	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	2.2
153	Hs.450230	NM_000598	IGFBP3	Insulin-like growth factor binding protein 3	2.2
272	Hs.9731	NM_002503	NFKB1B	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta	2.1
106	Hs.100431	NM_006419	CXCL13	Chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	2.1
156	Hs.321045	NM_014002	IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	2.1

Position	UniGene	RefSeq No	Symbol	Description	Fold changes
251	Hs.268675	NM_005587	MEF2A	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	2.1
221	Hs.409523	NM_002286	LAG3	Lymphocyte-activation gene 3	2.1
143	Hs.515126	NM_000201	ICAM1	Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	2.1
24	Hs.437877	NM_020547	AMHR2	Anti-Mullerian hormone receptor, type II	2.1
47	Hs.272493	NM_004167	CCL15	Chemokine (C-C motif) ligand 15	2.0
286	Hs.1976	NM_002608	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene)	2.0
235	Hs.465087	NM_005904	SMAD7	SMAD, mothers against DPP homolog 7 (Drosophila)	0.5
75	Hs.2259	NM_000073	CD3G	CD3G antigen, gamma polypeptide (TIT3 complex)	0.5
195	Hs.68876	NM_000564	IL5RA	Interleukin 5 receptor, alpha	0.5
263	Hs.529244	NM_003581	NCK2	NCK adaptor protein 2	0.5
325	Hs.170009	NM_003236	TGFA	Transforming growth factor, alpha	0.4
200	Hs.624	NM_000584	IL8	Interleukin 8	0.4
274	Hs.2764	NM_005007	NFKBIL1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 1	0.4
347	Hs.211600	NM_006290	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	0.4
242	Hs.145605	NM_006609	MAP3K2	Mitogen-activated protein kinase kinase kinase 2	0.4
181	Hs.126256	NM_000576	IL1B	Interleukin 1, beta	0.4
215	Hs.525704	NM_002228	JUN	V-jun sarcoma virus 17 oncogene homolog (avian)	0.3

**Table 3** Differentially expressed genes in uveitis patients confirmed by Real-time PCR

Up-regulated Genes			Down-regulated Genes		
Gene Symbol	Fold Up	RefSeq	Gene Symbol	Fold Down	RefSeq
<i>IL22</i>	16.5	NM_020525	CCL11	-3.4	NM_002986
<i>GDF5</i>	7.6	NM_000557	IL8	-3.7	NM_000584
<i>IL19</i>	7.0	NM_013371	<i>CXCL14</i>	-5.2	NM_004887
CX3CR1	6.1	NM_001337	CXCL2	-5.4	NM_002089
<i>IL20</i>	5.4	NM_018724	IL1B	-6.3	NM_000576
FASLG	3.2	NM_000639	IL6	-7.8	NM_000600
CCL21	3.0	NM_002989	CCL18	-11.9	NM_002988
<i>GDF10</i>	2.9	NM_004962	CSF2	-19.3	NM_000758
IL5	2.8	NM_000879	<i>CCL20</i>	-21.7	NM_004591
CCL19	2.6	NM_006274	<i>CCL7</i>	-24.0	NM_006273
CCR2	2.6	NM_000648			
LTB	2.5	NM_002341			
TNFSF10	2.5	NM_003810			
CXCL13	2.5	NM_006419			
IFNA2	2.3	NM_000605			
IL4	2.2	NM_000589			
<i>IL17E</i>	2.1	NM_022789			
IL16	2.1	NM_004513			