Association of Neovascular Age-Related Macular Degeneration with Specific Gene Expression Patterns in Peripheral White Blood Cells

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PURPOSE. Inflammation probably plays a major role in the pathogenesis of age-related macular degeneration (AMD). The authors evaluated whether AMD is associated with gene expression patterns in white blood cells (WBCs) and whether such a pattern may serve as a biomarker for the disease.

METHODS. Microarray analysis of gene expression in peripheral WBCs was performed on patients with neovascular AMD (NVAMD; n = 16) and controls (n = 16). Results were validated using quantitative real-time RT-PCR (QPCR) on another set of patients (n = 14) and controls (n = 16), respectively. QPCR findings were evaluated using receiver operator characteristic (ROC) curves and correlated with genotyping for the major risk single nucleotide polymorphisms (SNPs) for AMD in the genes for complement factor H and LOC387815.

RESULTS. NVAMD-associated expression was identified for eight sequences (false discovery rate [FDR] = 0%) and 167 sequences (FDR = 10%), respectively. There was an enrichment of genes involved in antigen presentation among the AMD-associated genes (P = 0.0029). QPCR confirmed increased expression (1.6- to 4.3-fold) of four genes (HSPA8, IGHG1, ANX1, and VKORC1) in association with NVAMD (P = 0.02-0.0002). Area under the curve for these genes according to ROC analysis ranged from 0.776 to 0.815. Gene expression was not associated with genotyping for risk SNPs or WBC counts.

CONCLUSIONS. NVAMD is associated with altered gene expression in peripheral WBCs that is not underlined by the major risk SNPs for the disease. Such altered expression may potentially serve as a biomarker for the disease. These data support the involvement of systemic immune response in the pathogenesis of AMD. (Invest Ophthalmol Vis Sci. 2010;51:53-58) DOI:10.1167/iovs.08-3019

Multiple types of evidence implicate both systemic and local inflammation in the pathogenesis of age-related macular degeneration (AMD). Among the evidence suggesting local inflammation in AMD are the presence of inflammatory mediators in drusen1,2 and the presence of macrophages in the choroid of eyes affected by AMD.3,4 Involvement of systemic inflammation in the disease is reflected by association of single nucleotide polymorphisms (SNPs) in complement components such as factor H, C3, C2, and B factor and SNPs in a chemokine receptor and, potentially, Toll-like receptor-5.5-13 Furthermore, antiretinal autoantibodies are present in the sera of patients with AMD,14-16 and altered plasma levels of inflammatory markers such as C-reactive peptide and complement components may also be associated with AMD.17-19 In addition, perturbed macrophage function is thought to lead to the development of features resembling AMD in mice strains deficient in chemokine receptors or their ligands.20,21 Macrophages can also modulate the development of neovascularization in mice models of laser-induced choroidal neovascularization.22-24

Microarray analysis of gene expression in blood cells detected disease-specific expression patterns in several pathologic conditions—among them lupus,25 multiple sclerosis,26 stroke,27 schizophrenia,28 and Huntington's disease29—in which inflammation may play a role. Characterizing such expression signatures may provide insight into the pathogenesis of the disease and may potentially serve as a surrogate biomarker for diagnosis and management of the disease.

In view of the data suggesting the involvement of inflammation and white blood cells (WBCs) in the pathogenesis of AMD, it is conceivable that gene expression signature in WBCs may also exist in AMD and that it may reflect the involvement of these cells in the disease. To that end, we have characterized gene expression patterns in WBCs from patients with AMD. The feasibility of using expression patterns as a biomarker for neovascular AMD (NVAMD) was then evaluated and compared with that of using major risk SNPs for the disease for the same purpose.

MATERIALS AND METHODS

Patients

Blood samples were drawn from patients with NVAMD and controls older than 60 years who were evaluated in the Department of Ophthalmology of the Hadassah-Hebrew University Medical Center in Jerusalem, Israel, for routine eye examinations or for pathologic conditions other than AMD. The authors adhered to the tenets of the Declaration of Helsinki. Institutional Ethics Committee approval was obtained, and each patient signed an informed consent form. AMD was diagnosed and graded according to the AREDS trial classification,30 and choroidal neovascularization was diagnosed based on ophtalmoscopy and fluorescein angiography. Only patients with active choroidal neovascularization according to fluorescein angiography were included in the study, and none of the patients had subretinal or subretinal pigment epithelium hemorrhage larger than 50% of the lesion size. Mean lesion size according to fluorescein angiography was 4057 ± 1411 μm (range, 1600-7700 μm). Inclusion criteria for the control group included clear media that enabled ophtalmoscopy and absence of intermediate-size drusen, multiple small drusen, or retinal pigment...
epithelial abnormalities characteristic of AMD (AREDS category I). Persons with severe systemic diseases such as malignancies, active ischemic heart disease, uncontrolled diabetes or pulmonary disease, or autoimmune diseases were excluded from the study and the control groups.

**White Blood Cell Separation and RNA Extraction**

Four milliliters of blood placed in tubes containing EDTA was used for WBC separation and complete blood count (CBC). CBC was performed at the central laboratory of the Hadassah Medical Center using an automated system. For RNA extraction, 8 mL hypotonic lysis buffer (155 mM NH₄Cl [Gadot, Or Akiva, Israel], 10 mM CH₃O₂H · NH₃ [Sigma-Aldrich, St. Louis, MO], 0.1 mM EDTA [J.T. Baker, Phillipsburg, NJ], pH 7.4) was added to the blood. The sample was stored on ice for 10 minutes and was then subjected to centrifugation at 2000g at 4°C for 10 minutes. Supernatant was discarded, and the previous stage was repeated. The pellet of white blood cells was resuspended in 1 mL reagent (TRI; Sigma-Aldrich). Total RNA was extracted according to the manufacturer’s instructions. Possible remnants of DNA were degraded (DNA-free; Ambion, Austin, TX), and RNA samples were purified (RNAeasy MinElute Cleanup Kit; Qiagen, Hilden, Germany). Samples were then stored at −80°C until further use.

**Microarray Analysis**

Microarray experiments were performed as we have previously described. Briefly, an indirect fluorescence-labeling method was applied using 20 µg purified RNA as a template for cDNA synthesis with reverse transcriptase (SuperScript II; Invitrogen, Karlsruhe, Germany) and incorporation of aminooxy-UTP (Sigma-Aldrich) during first-strand cDNA synthesis followed by coupling monoreactive Cy3 or Cy5 fluorescent dye (Amersham Biosciences Inc., Piscataway, NJ). The total amount of dye incorporation (measured in picomoles of dye per probe) and the ratio of unlabeled to fluorescence-labeled nucleotide in the probe were assessed by measuring probe absorbance at 260 nm, 550 nm, and 650 nm to assess DNA, Cy3, and Cy5 concentrations, respectively.

A reference sample design was applied by which sample (either NVAMD or control) RNA was labeled with Cy3 and reference RNA was labeled with Cy5. The analysis included 32 microarrays (16 NVAMD and 16 controls). Four patients underwent photodynamic therapy (PDT) >2 months before blood drawing for the study. A human array generated from an oligonucleotide set by Operon Biotechnologies Inc. (Cologne, Germany), version 3.0, which contains 35,035 oligonucleotide probes representing approximately 25,100 unique genes, was used for all experiments. The fluorescent probe was placed on the slide and was then incubated at 42°C for 16 to 22 hours. Posthybridization washes were performed, followed by scanning with the Axon scanner and the image analysis software (GenePix Pro 4.1 Microarray; Axon Instruments Inc., Union City, CA).

Microarray analysis, including background correction, filtration, and normalization, was performed using a microarray software (TM4) package developed by the TIGR Institute (Rockville, MD). Following by analysis using the MIDAS (Microarray Data Analysis System) program. SAM (Significance Analysis of Microarrays) algorithm and LIMMA (Linear Models for Microarray data) algorithm through the R program (http://www.r-project.org/) were used to assess significance. Both algorithms provide FDR (false discovery rate) as an estimate of the significance of the results.

Functional annotation of genes represented on the array was performed using Web-based software (http://bioinfo.vanderbilt.edu/gohtm; http://fatigo.bioinfo.cipf.es). Potential enrichment of specific functional classes among genes with significantly high or low expression levels associated with AMD was then assessed using Fisher’s Exact test.

**Quantitative Real-Time RT-PCR**

A first-strand synthesis kit (Reverse-IT; Allgene, Epsom, UK) was used to prepare cDNA from 1 µg total mRNA through reverse transcriptase polymerase chain reaction using anchored oligo dT primers. The expression levels of four genes—ANXA5, HSPA8, IGHG1, VKORC1—were assessed in WBCs from 14 patients with NVAMD and 16 controls that had not been analyzed by microarray. Eleven patients underwent PDT >2 months before blood drawing. GAPDH served as the endogenous control to which each sample was normalized. Reactions were performed using either the dye chemistry (SYBR Green; Applied Biosystems, Foster City, CA) or the fluorogenic 5′ nucleic acid (TagMan; Applied Biosystems) technique (Supplementary Table S1, http://www.iovs.org/cgi/content/full/51/5/DC1). Each tube contained 10 µL PCR mix and 2.8 µL primers for SYBR Green or 1 µL TaqMan assay. Optimal amounts of cDNA were calibrated for each primer. A total volume of 20 µL was completed by double distilled water. Samples were prepared in triplicate, and calculations were performed on the average value. Reactions were carried out and analyzed (ABI Prism 7000 and 7900HT systems; Applied Biosystems).

**Genotyping**

DNA was extracted from 200 µL whole blood using a DNA kit (FlexiGene; Sigma) according to the manufacturer’s protocol. Subjects who were studied using QPCR were genotyped for the LOC387715 rs10490924 and complement factor H (CFH) rs1061170 SNPs. A population of 163 patients with AMD and 104 controls was also genotyped for the VKORC1_2255 polymorphism. Genotyping was performed using restriction enzyme analysis of PCR (for rs10490924 and VKORC1_2255) or sequencing (for CFH) (Supplementary Table S2, http://www.iovs.org/cgi/content/full/51/5/DC1). Fragments were resolved on 2% agarose gel and visualized by ethidium bromide marking under an ultraviolet light lamp.

**ROC Analysis**

Receiver operating characteristic (ROC) curves assist in evaluating the feasibility of using a certain test for diagnostic purposes by measuring the area under the ROC curve (AUC). The closer the AUC is to 1, the more specific and sensitive is the test. A ROC curve was calculated for each of the gene expression assays evaluated by QPCR and for the genotyping results of LOC387715 and the CFH SNPs. Calculations were performed with Web-based software (http://www.rad.jhmi.edu/jeng/javarad/roc/JROCFITi.html). For input data purposes, patients with AMD were defined as “1” and controls as “0.” When evaluating gene expression, the RQ values (relative expression derived from QPCR) of individuals in each group were entered and processed. For the evaluation of genotypes, homozygotes for the wild-type allele were categorized as “1,” heterozygotes as “2,” and homozygotes for the polymorphism as “3,” and data were processed using an algorithm appropriate for discrete parameters.

**Statistical Analysis**

Statistical analysis for associations was performed using the χ² test. For all other comparisons the Student’s ttest was used. All tests were performed using biostatistics software (InStat; GraphPad, San Diego, CA).

**RESULTS**

**Microarray and QPCR Analysis**

Microarray and statistical analysis using the SAM algorithm identified eight sequences showing altered expression in WBCs in patients with NVAMD (mean age, 79.4 ± 6.9 years; range, 65–91 years) compared with age-matched controls (mean age, 75.7 ± 6.8 years; range, 64–90 years) at an FDR of 0%. Each of these sequences demonstrated increased expression in patients. There were 159 additional sequences showing altered expression at a less stringent significance level of 10% (data not shown). When implementing an alternative analysis algorithm, LIMMA, 53 differentially expressed sequences were
identified at an FDR of 20% after adjusting the P values for multiple comparisons using the Benjamini-Hochberg algorithm. Of these sequences, 33 were also identified by the SAM analysis at FDR 10%. Table 1 shows the 16 known genes from the 33 sequences identified by both algorithms.

QPCR validation experiments were performed on samples from 16 controls and 14 patients with NVAMD that had not been evaluated by the microarrays. Three genes (ANXA5, VKORC1, IGHG1) identified by both the SAM and LIMMA algorithms as having NVAMD-associated expression were included in this analysis, as was one gene identified only by SAM algorithms as having NVAMD-associated expression. These genes were selected for QPCR analysis because their known function suggests them as candidates for involvement in the pathogenesis of AMD. QPCR showed significantly higher mRNA levels in patients with NVAMD for each of the genes ranging from 1.6-fold to 4.3-fold compared with the controls (Table 1). Genes included in this group were HLA-DQA1, HLA-DQA2, and HLA-DQB1. These genes showed increased mRNA levels in patients with AMD compared with controls ranging from 1.6- to 1.8-fold.

**Correlation of Blood Counts and Genetic Variation with Gene Expression Levels**

Blood counts and genotyping for major risk SNPs for AMD were performed to assess whether differences in numbers of WBCs between patients and controls or the risk SNPs for the disease underlie gene expression alterations. There was no significant difference in the average (±SD) number of WBCs (8.5 ± 2.7 vs. 7.2 ± 1.8), lymphocytes (2.7 ± 2 vs. 2 ± 0.8), monocytes (0.6 ± 0.2 vs. 0.6 ± 0.3), granulocytes (5.4 ± 2 vs. 4.5 ± 1.4), eosinophils (0.2 ± 0.2 vs. 0.2 ± 0.1), or basophils (0.014 ± 0.036 vs. 0.057 ± 0.064) between patients with NVAMD and controls, respectively.

Although the SNPs in complement factor H (rs1061170) and LOC387715 (rs10490924) are associated with AMD in the Israeli population, there was no association between mRNA levels in WBCs of the four genes tested by QPCR and genotyping of the SNPs (Fig. 2).

**Receiver Operating Characteristic Curve Analysis**

To assess the feasibility of using measurements of mRNA levels of differentially expressed genes in WBCs as biomarkers for...
cies were similar in both populations (data not shown).

In this study, we genotyped 163 patients with NVAMD and 104 unaffected controls. There was no association of this SNP with NVAMD, we genotyped 163 patients with coronary heart disease and stroke.40 To evaluate potential risk SNPs for the disease, Measurements of altered gene expression in WBCs facilitated a noninferior distinction between patients with NVAMD and controls compared with that obtained by using genotyping for major risk SNPs for the disease.

Many of the genes that showed AMD-associated expression are involved in inflammation, a process that has a major role in the pathogenesis of AMD.41 For example, mRNA for the immunoglobulin heavy chain gamma 1 gene was increased in AMD in both microarray and QPCR analysis. This gene encodes the Fc area of the IgG antibody, the most abundant antibody in the serum and a major participant in the adaptive immune response. Such increased expression levels of IgG are in accordance with reports of higher levels of antiretina autoantibodies in retinas and sera of patients with AMD compared with controls,42–45 and of the presence of immunoglobulins within drusen, the hallmark of AMD.46,47

Functional analysis showed enrichment for genes involved in antigen presentation among genes demonstrating AMD-associated expression. These polymorphic genes, essential for regulation of the immune response, are involved in a variety of inflammatory and autoimmune diseases. Specific alleles of such HLA genes have been associated with the risk for AMD.44 Abnormal antigen presentation of retinal or drusen composites may contribute to the pathogenesis of AMD by fueling the self-inflicted attack of the immune system against the retina.

Increased levels of ANXA5 mRNA transcripts were also found in the WBCs of patients with AMD. ANXA5, which plays a role in the regulation of blood clotting, has been found in atherosclerotic plaques45 and is proposed to have anti-inflammatory functions.46 Interestingly, other annexins were previously identified in drusen.47 ANXA5 levels may increase in AMD as part of a healing response.

VKORC1 mRNA levels were also increased in the WBCs of patients with AMD. The product of this gene is an enzyme that activates vitamin K, an essential cofactor in many stages of the clotting cascade, and is involved in angiogenesis.48 A SNP in the encoding gene was associated with coronary heart disease and stroke,49 yet genotyping failed to identify an association between this SNP and NVAMD. Extensive cross-talk between the coagulation and complement systems is known to exist.49 Thus, the altered expression of genes involved in coagulation in the WBCs of patients with AMD, as we have described, may be of importance in the context of complement activation, angiogenesis, and hemorrhaging, all of which are characteristics of AMD. Altered expression of genes involved in angiogenesis, such as VKORC1, in the WBCs of patients with NVAMD supports the idea that subpopulations of WBCs, such as macrophages, may modulate the growth of choroidal neovascular vessels in patients with NVAMD.50–52 SNPs in several genes have been associated with the risk for AMD. Yet we show that altered gene expression in WBCs is not associated with major risk SNPs for AMD in complement factor H and on chromosome 10q26 (LOC387715) and that such expression patterns distinguish between patients and controls at least as well as genotyping for the risk SNPs. These data suggest that though polymorphisms in genes involved in the process may in part account for the pathogenesis of the disease, other factors also play important roles in AMD.

The existence of specific gene expression patterns in the WBCs of patients with AMD—in addition to the insight they provide to the pathogenesis of the disease—may serve as a biomarker for AMD. AMD is often undiagnosed until the late stages and is then often associated with substantial visual loss.53 Diagnosis of the disease at an earlier stage may facilitate the commencement of treatment and periodic follow-up that can improve visual outcome for patients with AMD.54 There is also a need for biomarkers that will correlate with disease progression and response to therapy. Thus, a blood test that facilitates detection of such biomarkers may serve as an important tool in the treatment algorithm for AMD.
Although several biomarkers for the disease have been suggested—among them measurement of inflammatory mediators and markers for oxidative injury in the serum—such markers were not demonstrated to be of value for the diagnosis of AMD in a clinical setting. Further research is required to assess whether measurement of gene expression will be useful for this purpose and to evaluate whether altered gene expression in WBCs reflects the involvement of these cells in NVAMD or is secondary to the disease process or the therapeutic interventions some patients underwent before they were enrolled in the study.

**References**
