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Molecular diagnosis of orbital inflammatory disease

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ABSTRACT

Orbital inflammatory diseases include thyroid eye disease (TED), granulomatosis with polyangiitis (GPA), sarcoidosis, and nonspecific orbital inflammation (NSOI). Histopathological diagnosis usually relies on the clinical context and is not always definitive. Gene expression profiling provides diagnostic and therapeutic information in several malignancies, but its role in evaluating nonmalignant disease is relatively untested. We hypothesized that gene expression profiling could provide diagnostic information for NSOI. We collected formalin-fixed, paraffin-embedded orbital biopsies from 10 institutions and 83 subjects including 25 with thyroid eye disease, 25 nonspecific orbital inflammation, 20 healthy controls, 6 with granulomatosis with polyangiitis, and 7 with sarcoidosis. Tissues were divided into discovery and validation sets. Gene expression was quantified using Affymetrix U133 Plus 2.0 microarrays. A random forest statistical algorithm based on data from 39 probe sets identified controls, GPA, or TED with an average accuracy of 76% ($p = 0.02$). Random forest analysis indicated that 52% of tissues from patients with nonspecific inflammation were consistent with a diagnosis of GPA. Molecular diagnosis by gene expression profiling will augment clinical data and histopathology in differentiating forms of orbital inflammatory disease.

1. Introduction

Molecular diagnosis using transcriptomics has demonstrated tremendous utility in malignant diseases (Hoadley et al., 2014) such as forms of lymphoma (Bohen et al., 2003; Dave et al., 2006) or ocular melanoma (van Gils et al., 2008). The added diagnostic value of profiling gene expression in nonmalignant diseases is less certain, but utility has been reported in inflammatory disorders such as myocarditis (Lassner et al., 2014), synovitis (Yeremenko et al., 2013), and esophagitis (Wen et al., 2013).

Orbital inflammation is an important cause of morbidity that results in pain, diplopia, and sometimes visual loss. Orbital biopsy helps to distinguish malignancy or infection from inflammation. Graves disease is the most common cause of orbital inflammation. It is often referred to as thyroid eye disease (TED). Additional systemic disease associations with orbital disease include sarcoidosis, granulomatosis with polyangiitis (GPA) (formerly Wegener's granulomatosis), sarcoidosis, Crohn's disease, IgG4-related disease, and histiocytosis. Many patients suffer from an inflammatory process that is difficult to categorize. These patients are labeled with terms such as nonspecific orbital inflammation (NSOI), orbital pseudotumor, or idiopathic orbital inflammation. Many of these diagnoses are suggested by clinical context and difficult to make based on histopathology alone. For example, the inflammatory infiltrate in TED can be scant such that, when TED affects orbital fat, it is sometimes difficult to distinguish TED from normal orbital adipose tissue. The diagnosis of GPA requires a vasculitis affecting a medium size vessel, but vessels of this size are rarely obtained on an orbital biopsy. Many patients suffer from an inflammatory process that is difficult to categorize. These patients are labeled with terms such as nonspecific orbital inflammation (NSOI), orbital pseudotumor, or idiopathic orbital inflammation.

While orbital inflammation is not rare, many patients do not undergo biopsy which entails some morbidity and expense. In order to understand nonspecific orbital inflammation, we organized an international consortium of orbital surgeons and pathologists. We have recently reported on the transcripts expressed by tissue representing the four most common diagnoses: TED, sarcoidosis, GPA, and NSOI (Rosenbaum

Abbreviations: GPA, granulomatosis with polyangiitis; NSOI, nonspecific orbital inflammation; TED, thyroid eye disease.

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et al., manuscript submitted). In this report, we test the hypothesis that a diagnostic algorithm based on a limited number of transcripts could complement observations made by experienced pathologists.

2. Methods

2.1. Centers, biopsies, database

This study was approved by the Institutional Review Board (IRB) at Oregon Health & Science University (IRB00006301) and at each of the other contributing centers. The research adhered to the tenets of the Declaration of Helsinki. Formalin-fixed, paraffin-embedded (FFPE) samples and relevant demographic and clinical data were obtained from 10 institutions. The diagnoses of NSOI, sarcoidosis, GPA, TED, and normal were based on the clinical and histopathological information obtained and submitted by orbital disease specialists and ocular pathologists from their respective institutions. All biopsies were further reviewed by two of the authors (DJW and HEG) as noted below.

Biopsies of the orbital adipose tissue from a total of 83 subjects were studied (20 controls with no known orbital disease, 25 with NSOI, 6 with GPA, 7 with sarcoidosis, 25 with TED). The age, gender, diagnoses, and clinical information supporting each diagnosis has been reported elsewhere (Rosenbaum et al., manuscript submitted).

2.2. Pathology review

Two ocular pathologists (D.J.W. and H.E.G.) independently evaluated hematoxylin and eosin stained slides of all samples for histopathological characteristics without reference to the indications for biopsy or other clinical information. After rendering a diagnosis in a masked fashion, the pathologists were informed of the diagnosis from the institution where tissue had been obtained. In some cases, additional stains were requested or additional clinical information was reviewed. A few cases with an ambiguous diagnosis were excluded. In all cases included in this study, the diagnosis reached by Drs. Wilson and Rosenbaum agreed with the contributing center's diagnosis. Thus, a consensus diagnosis which combined clinical and histopathological information was considered the gold standard to which we compared the diagnoses rendered by either computer algorithm or by an expert pathologist.

2.3. Tissue preparation and gene expression profiling

Prior to gene array analysis, the tissues were roughly evenly divided on the basis of time of receipt at OHSU. This created an initial discovery set and a validation set. RNA extraction and microarray assays were performed in the OHSU Gene Profiling Shared Resource. For each specimen, multiple 10–20 μm sections were collected and total RNA was extracted with the miRNeasy FFPE kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was amplified and labeled with SensationPlus FFPE Amplification and 3′ IVT Labeling kits (Affymetrix, Santa Clara, CA) for microarray analysis. For the majority of samples, an input of 50 ng of RNA was used, and a minimum input of 20 ng RNA was used for samples with limiting RNA recoveries. The standard Affymetrix protocol was followed for hybridizing biotin-labeled cDNA targets with a GeneChip Human Genome U133 Plus 2.0 array (Affymetrix, Santa Clara, CA). The Human U133 Plus 2.0 array contains over 54,000 probe sets for 47,000 human transcripts and variants. Following hybridization, arrays were processed and stained according to standard Affymetrix protocols, then scanned on the GeneChip Scanner 3000 7G system (Affymetrix). Affymetrix GeneChip Command Console (AGCC) v. 3.1.1 and Affymetrix Expression Console v. 1.1 software were used for image processing and expression analysis for initial quality control, respectively.

2.4. Statistical analysis

Affymetrix CEL files of each set were preprocessed separately by the Robust Multiarray Analysis (Irizarry et al., 2003). The posterior logarithm of odds that the gene is differentially expressed (Smyth, 2004) when comparing GPA or TED samples versus uninflamed controls were used to select probe sets for principal coordinate plots (Mardia, 1978) and to classify NSOI samples into non-NSOI disease groups by using random forests, an ensemble learning method for classification (Breiman, 2001). Prediction accuracies by random forest and by pathologists were compared by confusion matrices (Kohavi and Provost, 1998). Principal coordinate analyses of the selected probe sets were employed for graphical presentation. Statistical computing was performed with 'affy', 'caret' 'MASS', 'limma' and 'randomForest' packages in the R project (http://www.r-project.org).

3. Results

NSOI is heterogeneous in symptoms, histopathology, and response to treatment. The extreme variability of NSOI histopathology is illustrated in Fig. 1. Tissue can vary markedly in terms of the degree of inflammation, the extent of uninflamed adipose tissue, the amount of fibrosis, and the presence of granuloma.

Tissues were divided based on the time of collection into a discovery set and a validation set. We previously reported the heterogeneity of gene expression in NSOI tissues and the clustering of the other diseases based on analyses that included all probe sets with significantly different signals for disease versus uninflamed control comparisons in both data sets (Rosenbaum et al., manuscript submitted). In this current study, we distinguish the disease groups based on a minimal number of probe sets. To visualize the complex profiles, we employed a principal coordinate analysis (PCA). In a PCA, the relative similarity of transcriptomes of two samples is indicated by their proximity on a graph.

The previously reported clustering (manuscript submitted) was replicated with a small subset of the data (Fig. 2). For each comparison of TED or GPA versus normal, 20 top-ranked probe sets were selected according to the posterior logarithm of odds that the gene is differentially expressed (Smyth, 2004). One probe set was common to both lists, so the combined list included 39 probe sets. PCA plots based on the 39 selected probe sets for the all of the samples with a consensus diagnosis of control, TED, GPA or sarcoidosis from set 1 or set 2 are shown in Fig. 2A and B, respectively. The clustering of samples representing normal tissue indicates the relative similarity of the tissue. The samples from subjects with TED are also relatively homogeneous and have the closest proximity to normal samples among the four disease groups. The gene expression profiles from subjects with either sarcoidosis or GPA also tend to cluster. Fig. 2C and D shows the same PCA plots with the addition of the NSOI samples from the respective data sets. The spatial relationships depicted in Fig. 2C and D are better appreciated in videos (Discovery set PCA video.MP4 and Validation set PCA video.mp4) since the relationships are 3 dimensional. The gene expression profile for patients with NSOI showed more heterogeneity than the other four categories consistent with the hypothesis that NSOI is not a single disease entity. The NSOI sample that clustered with sarcoidosis contained non-caseating granuloma on histopathology. A diagnosis of sarcoidosis was not made because the patient had no pulmonary symptoms and neither a chest x-ray nor a chest CT scan was obtained.

Two ophthalmic pathologists were asked to render a histological diagnosis in the absence of clinical information. Both pathologists correctly identified the 7 tissues from patients with sarcoidosis on the basis of non-caseating granulomas. The pathologists were not asked to distinguish between NSOI and TED due to the absence of discriminating features. For these cases, the pathologists were credited with the correct diagnosis if the consensus diagnosis based on both histopathology and clinical data was either NSOI or TED.