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Research Paper

Molecular signatures define alopecia areata subtypes and transcriptional biomarkers

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ABSTRACT

Alopecia areata (AA) is an autoimmune disease typified by nonscarring hair loss with a variable clinical course. In this study, we conducted whole genome gene expression analysis of 96 human scalp skin biopsy specimens from AA or normal control subjects. Based on gene expression profiling, samples formed distinct clusters based on the presence or absence of disease as well as disease phenotype (patchy disease compared with alopecia totalis or universalis). Differential gene expression analysis allowed us to robustly demonstrate graded immune activity in samples of increasing phenotypic severity and generate a quantitative gene expression scoring system that classified samples based on interferon and cytotoxic T lymphocyte immune signatures critical for disease pathogenesis.

1. Introduction

Alopecia areata (AA) is an autoimmune skin disease in which the hair follicle is the target of immune attack. Patients characteristically present with round or ovoid patches of hair loss usually on the scalp that can spontaneously resolve, persist, or progress to involve the scalp or the entire body (Gilhar et al., 2012). The three major phenotypic variants of the disease are patchy-type AA (AAP), which is often localized to small areas on the scalp or in the beard area, alopecia totalis (AT), which involves the entire scalp, and alopecia universalis (AU), which involves the entire body surface area. There are currently no FDA approved drugs for AA. Treatment is often empiric and typically involves observation, intralesional steroids, topical immunotherapy or broad immunosuppressive treatments of variable efficacy. The more severe forms of the disease, AU and AT, are often recalcitrant to treatment. Despite its high prevalence and the need for effective treatments, the molecular and cellular effectors of AA have not been well studied. It is currently unclear if distinct pathogenic mechanisms drive these more severe forms of the disease, or whether those disease mechanisms are exacerbated in AU and AT compared to AAP.

Histologically, AA is characterized by an immune infiltrate centered around the hair bulb. This infiltrate is made up of predominantly CD4 and CD8 T cells (Ito et al., 2008), although other cell types, including natural killer cells (Ito et al., 2008; Kaufman et al., 2010), macrophages (Castellana et al., 2014), mast cells (Bertolini et al., 2014) and eosinophils (Elston et al., 1997) may also be present. Substantial differences in histological appearance have not been described when comparing AAP, AT, and AU samples, although others have cited that disease duration may impact the amount of peribulbar infiltrate, with more acute cases being reported as having relatively more robust inflammation and chronic cases having less (Whiting, 2003a).

Recent strides in the field have transformed our understanding of disease pathogenesis, drug targets, and potential therapeutic solutions. The results of our initial genome wide association study (GWAS) (Petukhova et al., 2010) and, more recently, of a large GWAS meta-analysis (Betz et al., 2015) have identified numerous loci that imply a strong role for variants in genes that direct and influence immune responses. Interestingly, almost all of the implicated immune genes have been associated with other autoimmune diseases, including type 1 diabetes, rheumatoid arthritis, and celiac disease, lending further support for the common-cause hypothesis of autoimmune diseases (Gregersen and Olsson, 2009). Of particular note, single nucleotide polymorphisms in the ULBP3 and ULBP6 genes confer an increased risk for developing the disease and are uniquely associated with AA.

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The ULBP family of genes encodes proteins that serve as ligands for NKG2D and, when expressed, mark a cell for immune targeting by natural killer cells or NKG2D-expressing CD8 T cells. These data led to the recognition of NKG2D-bearing CD8 T cells in the peribulbar infiltrate in skin sections of lesional scalp biopsy specimens of patients with AA as well as in affected skin and skin-draining lymph nodes from the C3H/HeJ mouse model of spontaneous AA (Petukhova et al., 2010; Xing et al., 2014). Adoptive transfer of this population of cells from C3H/HeJ mice with alopecia into unaffected C3H/HeJ mice led to the induction of alopecia, substantiating a pivotal role for these effector cells in the mouse AA model (Xing et al., 2014).

We previously identified prominent interferon (IFN) and common gamma chain cytokine (γc) signatures in AA, both of which we postulated contributed to disease pathogenesis (Xing et al., 2014). Based on these findings, a therapeutic strategy based on inhibition of critical members of a family of signaling molecules, Janus kinases (JAKs), was found to be effective at treating AA in a mouse model of disease and a small series of human patients. Gene expression profiling played a critical role in our selection of small molecule JAK inhibitors for AA, and we reasoned that gene expression studies that include the different AA phenotypes have the potential to provide additional insights into novel therapeutic solutions as well as pathogenic mechanisms.

Here, we profiled scalp biopsy samples collected from a total of 96 patients with a range of AA phenotypes and normal control patients. Patient samples were collected from the National Alopecia Areata Registry sites across the United States after phenotypic classification by dermatologists who specialize in hair disorders. Skin biopsy samples were then interrogated using microarray-based gene expression analysis to identify the AA-specific gene expression signature. We found a striking level of immune activity in AT/AU samples by gene expression analysis. Despite the lack of consistently effective treatments in AT and AU, these data suggest that drugs that disrupt this immune activity may be useful for therapeutic purposes. Furthermore, based on our data, we created an Alopecia Areata Disease Severity Index (ALADIN), a gene expression metric that effectively distinguishes AT/AU samples, AAP samples, and normal control (NC) samples from each other. ALADIN may be used to accurately track disease activity in patients undergoing conventional or experimental treatments.

2. Materials and Methods

2.1. Experimental Design

The objective of this study was to identify immune and nonimmune signaling pathways as well as biomarkers in the affected skin from patients with AA. The overall design was to use whole genome based gene expression techniques on skin samples from patients with AA of variable severity and compare those with skin samples from healthy controls. Sample collection, sample processing and data analysis are described below.

2.2. Human Patient Demographics

Two independent datasets were collected from four National Alopecia Areata Foundation (NAAF) registry sites. Our discovery dataset consisted of samples from 63 patients (20 AAP, 20 AT/AU, and 23 normal controls). Our validation dataset was comprised of samples from 33 patients (8 AAP, 12 AT/AU, and 13 Normal controls). A more complete description of the datasets broken down by disease status, gender, age, and NAAF registry site is provided in Supplemental Table 1.

2.3. Ethics Statement

All studies have been approved by the Institutional Review Boards at the Columbia University Medical Center, the University of Minnesota, the University of California, San Francisco, and the M.D. Anderson Cancer Center and were conducted under the Declaration of Helsinki principles. Informed written consent was received from participants prior to inclusion in the study.

2.4. Human Tissue Sampling and Processing

Skin punch biopsy specimens were fixed in the PAXgene Tissue Containers and shipped overnight to Columbia University. Samples were bisected, with one half of the sample processed using the PAXgene tissue miRNA kit to extract RNA. Library prep was performed for microarray analysis using Ovation RNA Amplification System V2 and Biotin Encore kits (NuGen Technologies, Inc., San Carlos, CA). Samples were subsequently hybridized to Human Genome U133 Plus 2.0 chips (Affymetrix, Santa Clara, CA) and scanned at the Columbia University Pathology Core or the Yale Center for Genome Analysis.

Microarray data were deposited in Gene Expression Omnibus, accession GSE68801.

2.5. Analysis Packages

Quality control of microarrays was performed using the affyAnalysisQC package from http://arrayanalysis.org/. Differential expression in these studies was defined by an absolute fold change threshold of 1.5 with a Benjamini–Hochberg-corrected significance threshold of 0.05. Clustering and principal component analysis was done using the modules provided in the Bioconductor R package. Network images were generated with Cytoscape.

2.6. Microarray Preprocessing and Quality Control

Microarray preprocessing was performed using BioConductor in R. Preprocessing of the two datasets, discovery dataset (63 samples) and the validation dataset (33 samples), were performed separately using the same pipeline. Quality control was performed using the affyanalysisQC package from http://arrayanalysis.org/. The discovery dataset and the validation dataset were normalized separately using GCRMA and MAS5. The Affymetrix HGU-133Plus2 array contains 54675 probe sets (PSIDs). Filtering was performed so that PSIDs that were on the X or Y chromosome, that were Affymetrix control probe sets, or that did not have Gene Symbol annotation were removed from all arrays for further downstream analysis. For the 3D plot of the ALADIN scores, all 96 samples from both datasets were combined before performing GCRMA normalization and correcting for batch effects.

2.7. Sample Filtering and Batch Correction

In order to perform analysis on the 63 AA lesional (both AT/AU and AAP) and NC samples in the discovery data set, PSIDs were further filtered to remove PSIDs that had not been called present on at least one of the 63 arrays resulting in 36954 PSIDs. Correction for batch effects was performed using the implementation of the function ComBat available in the sva package with gender and AA group (AT/AU, AAP, and normal) used as covariates. No batch correction was required for the validation set.

2.8. Differential Expression Analysis

Differential analysis was performed on the batch corrected discovery data set using linear models as implemented in the limma package in Bioconductor (Smyth, 2004). Two-sample comparisons were performed separately to identify PSIDs differentially expressed in AA patients versus normal controls, in AAP patients versus normal controls, and in AT/AU patients versus normal controls treating gender as a fixed factor.