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Title: Gene expression profiling suggests severe, extensive central centrifugal cicatricial alopecia may be both clinically and biologically distinct from limited disease subtypes

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The natural history of central centrifugal cicatricial alopecia (CCCA) is widely variable. Some patients experience rapid progression to extensive, end-stage disease while others never approach extensive involvement over decades, suggesting heterogeneity in CCCA disease phenotype. To better characterize clinically severe disease in CCCA, tissue samples were obtained from the peripheral, hair bearing lesional scalp of women with clinically focal, limited, and extensive CCCA disease involvement. A microarray analysis was conducted to identify differential expression of genes previously identified to be preferentially expressed in the lesional scalp vs non-lesional scalp of CCCA patients. Clinically extensive, severe CCCA was characterized by increased expression of *MMP9*, *SFRP4*, and *MSR1* when directly compared with focal and limited disease. These biomarkers correspond to dysregulated pathways of fibrosis, Wnt signaling, and macrophage-mediated inflammatory processes, respectively. These findings hold significance for both possible targets for future study of prognostic markers of disease severity and new potential therapeutic targets. In summary, this study suggests clinically extensive, severe CCCA may have a differential gene expression pattern in the lesional scalp of affected patients, in addition to its clinical distinction.

Key words: alopecia; cicatricial alopecia; central centrifugal cicatricial alopecia; fibrosis; fibroproliferative disorders

Introduction

Central centrifugal cicatricial alopecia (CCCA) is a primary lymphocytic alopecia with highest prevalence among Black women.[1] Clinically, CCCA is characterized by hair loss that begins on the vertex of the scalp and progresses centrifugally, eventually leading to the replacement of healthy hair follicles with fibrous tracts.[2] While the exact mechanism leading to end-stage fibrosis has not been identified, several studies have provided insight into the potential genetic factors at play in disease development. The most recent breakthrough in the genetic underpinnings of CCCA was the discovery of *PADI3* variants in 24% of CCCA patients.[3] Genes implicated in the pathogenesis of fibroproliferative disorders have also been identified to be preferentially upregulated in the affected scalp tissue of CCCA patients in one study,[4] and an additional study highlighted women with CCCA are five times more likely to develop uterine fibroids compared to their unaffected counterparts.[5] These findings have led to one proposed theory that CCCA may resemble a group of disorders characterized by persistent inflammation

and impaired wound healing resulting in excessive fibrosis, collectively termed fibroproliferative disorders (FPDs).[6]

Though progress has been made in identifying potential factors involved in the development of CCCA, there are remaining gaps in the existing literature regarding the natural history of disease progression. In the authors' experience, clinical progression of hair loss is often variable, as some patients experience rapid progression to end-stage disease within a few years of disease onset while others may experience slow, gradual progression over decades and may never approach end-stage or extensive involvement. Though a recent study found that clinically unaffected areas of the scalp in CCCA patients have histologic evidence of active disease,[7] variation in disease course, particularly in the rate of disease progression, is unexplained by this discovery and instead suggests multiple CCCA disease phenotypes. We therefore sought to characterize the expression pattern in a small subset of CCCA patients with accelerated progression to clinically severe, extensive disease involvement.

Methods

This study was approved by the Johns Hopkins Hospital institutional review board. All patients underwent informed consent with authorization from the Johns Hopkins ethics board. Sixteen patients with CCCA were recruited between 2017 and 2020. Diagnostic confirmation of CCCA was achieved through histology in patients with early stage disease, and through clinical evaluation by at least two board-certified dermatologists in patients with end-stage disease. The clinical severity of disease was staged using a 6-point photographic scale with gradation ranging from normal hair density without hair loss (stage 0) to end-stage, fibrotic scalp with permanent hair loss (stage 5).[8] Patients were grouped into the following disease severity categories based on clinical examination at the time of tissue sample collection: focal (stage 1A through 2B), limited (stage 3A through 4B), and extensive (stages 5A and 5B) disease involvement (see Figure 1). A 4-mm punch biopsy was obtained from the peripheral, hair bearing areas of the affected vertex scalp in each patient. All patients were either treatment naïve or had not received treatment for at least 1 year at the time of tissue specimen collection.

RNA isolation and microarray analysis

Immediately following collection, scalp tissue samples were submerged in a 2-mL tube containing RNAlater stabilization reagent (QIAGEN, Valencia, CA). Samples were subsequently

stored at 4°C for 24 hours and then transferred to a -80°C freezer for long-term storage. Total RNA was extracted using a QIAGEN RNeasy fibrous tissue kit (QIAGEN, Valencia, CA). Standard protocols were followed for total RNA extraction and tissue homogenization. Prior to microarray analysis, RNA concentration and quality were measured with a bioanalyzer. Gene-level expression through a transcriptome-wide analysis of more than 20,000 well-annotated human genes was measured using the Human Clariom S Assays (Affymetrix, Santa Clara, CA). Differential gene expression between the focal, limited, and extensive disease groups was evaluated with the paired-sample one-way 2-tailed t-test to assess fold changes in gene transcript expression and statistical significance, as measured by P-values. Statistical significance was determined at $P \leq 0.05$. Batch effect was corrected for using a two-way analysis of variance (ANOVA), with batch date as the second degree of freedom.

Results

To focus only on genes formerly implicated as characteristic of the lesional scalp in CCCA patients, previously published microarray expression data from 2018 was re-analyzed.[5] Two-hundred genes with the highest fold change in the lesional compared to non-lesional scalp of CCCA patients were identified. Expression patterns of these 200 genes were then assessed based on disease severity. Of the 16 patients analyzed, six patients had focal disease, seven patients had limited disease, and three patients had extensive disease based on clinical examination at the time of tissue sample collection. Patients ranged from ages 27 to 70, with disease duration prior to biopsy ranging from months to decades. Patients with extensive CCCA had a median time from disease onset to stage 5A or 5B involvement of 5 years.

Gene expression patterns among 16 patients with focal, limited, and extensive CCCA were directly compared. Twelve genes were preferentially expressed in the lesional scalp of CCCA patients[5] and also consistently upregulated with increasing severity of disease (Table 1). Expression patterns in focal and localized disease were noted to be very similar across these 12 genes and patients were therefore further dichotomized into extensive or non-extensive disease groups. Five genes with the highest fold changes in extensive compared to non-extensive disease were identified. Notably, the three genes with the highest fold changes, matrix metalloproteinase 9 gene (*MMP9;* p=0.015), secreted frizzled-related protein gene (*SRFP4*; p=0.028), and macrophage scavenger receptor 1 gene (*MSR1*; p=0.011), have been implicated in FPDs or

disorders of aberrant fibrogenesis.[9,10,11] Lysozyme gene (*LYZ*), NCK associated protein 1 like gene (*NCKAP1L*), and doublecortin like kinase 1 gene (*DCLK1*), though not previously implicated in FPDs, were also shown to have increased expression in extensive disease with only the latter found to have no statistical significance (p=0.0240; p=0.026; p=0.100, respectively).

Conclusions and Perspectives

This study suggests that severe, extensive CCCA is potentially both clinically and biologically distinct, and is characterized by increased expression of *MMP9*, *SFRP4*, and *MSR1* in the lesional scalp of CCCA patients. These biomarkers correspond to dysregulated pathways of fibrosis, Wnt signaling, and macrophage-mediated inflammatory processes, respectively.

Matrix metalloproteinases (MMPs) are extracellular endopeptidases physiologically involved in extracellular matrix turnover and degradation.[12] Whereas some MMPs are known catalysts of fibrosis, others have an inhibitory role.[13] In excess, the presence of pro-fibrotic MMPs can lead to the destruction of the extracellular matrix and subsequently stimulation of inflammation. Our results reveals *MMP9*, which is thought to have a stimulatory role in fibrosis, as characteristic of severe disease in CCCA.[9] Increased expression of *MMP9* by neutrophils, fibroblasts, and alveolar epithelial cells in the lungs, elevated circulating blood levels, and upregulated expression in bronchoalveolar lavage fluid have all been implicated in idiopathic pulmonary fibrosis (IPF).[9,14] The alveolar macrophages in IPF are also thought to facilitate *MMP9* expression, further contributing to fibrosis.[15]

Our data also reveal a role for macrophage-mediated pro-inflammatory processes in CCCA. Specifically, we found increased expression of *MSR1* is associated with increased disease severity in CCCA. *MSR1* is a protein coding gene that has been implicated in several macrophage-mediated pathological processes such as Alzheimer's disease and atherosclerosis.[16,17] It plays a critical role in the induction of inflammatory reactions and immune responses.[18] *MSR1* has been implicated in the development of lung fibrosis in patients with IPF, with the number of MSR1-positive macrophages negatively correlated with FEV1 and FVC values, markers of lung function in these patients.[10]

Finally, increased expression of *SFRP4* was found in severe, extensive disease in CCCA. *SFRP4* acts as a soluble modulator of the Wnt signaling pathways, which have been implicated as a key mediator of hair follicle devleopment.[11,19] *SFRP4* has key function in wound healing and

fibrosis. Macrophages in late stage wounds phagocytize and degrade *SFRP4*, resulting in chronic Wnt activity that drives tissue fibrogenesis over regeneration.[11] Increased expression of *SFRP4* has also been found in the affected skin of patients with systemic sclerosis and is thought to contribute to skin fibrosis through non-canonical Wnt activation.[20,21] Mouse models have further examined the role of *SFRP4* and Wnt activation in wound healing, showing that increased Wnt activity in older wounds was associated with loss of hair neogenesis.[22] These findings suggest a potential connection between chronic activation of Wnt signaling and scarring precluding hair neogenesis.

In summary, our results highlight the differential gene expression profile of the lesional scalp in clinically severe CCCA. This holds significance in establishing potential targets of interest for future study in the identification of prognostic markers of disease severity. Notably, one patient with focal disease experienced the onset of hair loss 20 years prior to study evaluation without progression to extensive, end-stage fibrosis even in the absence of consistent treatment, whereas a patient with severe, extensive disease experienced progression to this stage within only 2 years of the onset of hair loss. This underscores that terms such as "early" and "late" disease may not fully encapsulate the natural history of CCCA. Rapidly progressive, extensive fibrosis in CCCA may not be the result of simply delayed treatment, but instead represent the biological heterogeneity of disease. The ability to distinguish patients at risk for a severe phenotype of CCCA with predisposition to relatively rapid progression to end-stage fibrosis is important for identifying patients warranting aggressive treatment.

Our results also indicate potential therapeutic targets for CCCA. For example, a synthetic MMP inhibitor, batimastat, led to significant reduction in the development of bleomycin-induced pulmonary fibrosis in mice and was specifically found to downregulate the expression of matrix metallopeptidase 2 gene (*MMP2*) and *MMP9*.[23] This therapy may hold promise for future clinical trials in CCCA patients. Future prospective studies should also aim to identify whether the differential molecular profile seen in patients with extensive disease is present at the onset of disease or becomes more apparent as the disease progresses. This information would allow a patient-specific approach to the treatment of CCCA, inclusive of both genetic and environmental factors at play. This study is limited by the small patient sample size at a single institution and use of whole scalp tissue for the microarray analysis.

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Data Availability Statement: The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE179054 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179054).

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Table 1. Twelve genes were found to be preferentially expressed in the affected scalp of CCCA patients and also consistently upregulated with increasing severity of disease. In most cases, log2 fold change approaches 1 when comparing focal to limited disease indicating similar expression patterns. † denotes the top 5 genes based on log2 fold change in extensive vs non-extensive disease

Gene symbol	Gene name	Log2 fold change in gene expression

		Limited vs focal	Extensive vs limited	Extensive vs focal	Extensive vs non-extensive disease	p-value*
MMP9†	Matrix metalloproteinase 9 gene	-1.19	3.80	3.19	3.64	0.015
SFRP4†	Secreted frizzled related protein 4	1.08	3.42	3.69	3.49	0.028
MSR1†	Macrophage scavenger receptor 1 gene	1.11	2.63	2.91	2.70	0.011
LYZ†	Lysozyme gene	-1.20	2.69	2.91	2.57	0.024
DCLK1†	Doublecortin like kinase 1 gene	-1.08	1.96	1.81	1.92	0.100
PLEK	Pleckstrin gene	1.13	1.85	2.10	1.91	0.070
NCKAP1L	NCK associated protein 1 like gene	1.23	1.81	2.22	1.91	0.026
ENPP1	Ectonucleotide pyrophosphatase 1 gene	1.81	1.64	2.97	1.90	0.085
ADAM12	ADAM metallopeptidase domain 12 gene	1.01	1.84	1.86	1.85	0.100
F13A1	Coagulation factor XIII A chain gene	1.34	1.51	2.02	1.62	0.159
PDE4DIP	Phosphodiesterase 4D interacting protein gene	1.36	1.36	1.85	1.40	0.147

PPBP	Pro-platelet basic protein gene	1.42	1.28	1.81	1.39	0.627

*p-value calculated for extensive vs non-extensive disease fold change in gene expression

Figure 1. Spectrum of CCCA disease severities: Patients with stage 1B (focal, a), stage 3B (limited, b) and stage 5B (extensive, c) disease

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