Received: 12 July 2018 | Revised: 23 April 2019 | Accepted: 9 May 2019

DOI: 10.1111/exd.13986

ORIGINAL ARTICLE

Experimental Dermatology WILEY

Integrative analysis of rare copy number variants and gene expression data in alopecia areata implicates an aetiological role for autophagy

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Funding information
National Institute of Arthritis and Musculoskeletal and Skin Diseases, Grant/Award Number: K08AR069111, P30AR069632, P50AR070588, R01AR065963, R01AR52579 and R01AR56016

Abstract

Alopecia areata (AA) is a highly prevalent autoimmune disease that attacks the hair follicle and leads to hair loss that can range from small patches to complete loss of scalp and body hair. Our previous linkage and genome-wide association studies (GWAS) generated strong evidence for aetiological contributions from inherited genetic variants at different population frequencies, including both rare mutations and common polymorphisms. Additionally, we conducted gene expression (GE) studies on scalp biopsies of 96 patients and controls to establish signatures of active disease. In this study, we performed an integrative analysis on these two datasets to test the hypothesis that rare CNVs in patients with AA could be leveraged to identify drivers of disease in our AA GE signatures. We analysed copy number variants (CNVs) in a case-control cohort of 673 patients with AA and 16 311 controls independent of the case-control cohort of 96 research participants used in our GE study. Using an integrative computational analysis, we identified 14 genes whose expression levels were altered by CNVs in a consistent direction of effect, corresponding to gene expression changes in lesional skin of patients. Four of these genes were affected by CNVs in three or more unrelated patients with AA, including ATG4B and SMARCA2, which are involved in autophagy and chromatin remodelling, respectively. Our findings identified new classes of genes with potential contributions to AA pathogenesis.

KEYWORDS
alopecia areata, autophagy, copy number variants, integrative genomics

1 | INTRODUCTION

Alopecia areata (AA) is a common autoimmune disorder with a prevalence of 2.1%.[1] In AA, an aberrant interaction between the immune system and the hair follicle leads to non-scarring hair loss that generally begins in patches on the scalp but may progress to affect the entire scalp (alopecia totalis; AT) and body (alopecia universalis; AU). A genetic basis for the disease was first suggested by studies in families and twin pairs that demonstrated an increased risk of disease among family members.[2] Our earlier genetic linkage studies in AA families provided definitive evidence for aetiological contributions from rare variants, with the identification of several genomic regions harbouring strong statistical evidence for co-segregation.[3] However, these regions identified by linkage were too large to

implicate specific genes in AA disease pathogenesis, and candidate genes were challenging to identify.

More recently, we conducted a genome-wide association study (GWAS) and a GWAS meta-analysis, which identified common variants (ie single nucleotide polymorphisms; SNPs) that are associated with AA across 14 genomic regions that are much smaller than linkage intervals (on the order of kilobases rather than megabases). Many of the GWAS regions implicated individual genes or small clusters of functionally related genes, thus providing new and clinically relevant insight into the disease.[4–6] For example, immune susceptibility loci included genes that are involved in regulatory T-cell activation and proliferation (cytotoxic T-lymphocyte-associated protein 4; CTLA4, interleukin 21; IL21, interleukin-2; IL2, interleukin-2 receptor alpha chain; IL2RA, suppressor of cytokine signaling 1; SOCS1), IFNγ-producing NKG2D-mediated cytotoxic T-cell activity (UL16-binding protein 3/6; ULBP3/6, interleukin 2; IL2), in addition to the human leucocyte antigen (HLA). Furthermore, we identified several genes that were expressed in the hair follicle, including genes involved in autophagy and pigmentation (syntaxin 17; STX17), oxidative stress (peroxiredoxin 5; PRDX5) and apoptosis (Bcl-2-like protein 11; BCL2L11, also known as BIM). These findings prompted us to initiate functional immunological and pharmacological studies that demonstrated IFNγ-producing CD8+NKG2D cytotoxic T cells are both necessary and sufficient to induce AA in a mouse model of the disease. Finally, we showed that targeting these cells with topical or systemic JAK inhibitors induces hair regrowth in patients with long-standing moderate-to-severe AA.[7–11] Our GWAS were highly fruitful and represented an unusual example of GWAS candidate genes and functional studies that led to new targeted treatment approaches.[12]

Despite these successes, here, we sought to return to the investigation of rare variants suggested by our linkage studies. We were compelled by findings in other chronic diseases that have found phenotypic contributions from genetic variants across the entire spectrum of allele frequencies.Importantly, genes that have been implicated in chronic diseases by the presence of rare causal mutations have proven to be relevant to a large number of patients who don't carry such mutations. For example, the PCSK9 gene was first identified by linkage studies of familial hypercholesterolaemia that identified rare causal mutations.[13] While very few patients with elevated LDL have monogenic mutations in PCSK9, broader population relevance was established when GWAS identified common variants in and around PCSK9 that increase risk of elevated LDL, myocardial infarction and coronary artery disease.[14] Importantly, clinical relevance was established when it was shown that targeting PCSK9 with a monoclonal antibody could lower blood LDL levels, offering a new treatment strategy for the 10%-20% of patients who are statin-intolerant.[15]

Thus, we revisited our earlier strategy focused on rare variants with the goal of integrating gene expression studies to streamline the identification of causal genes. In parallel with our genetic studies, we previously performed extensive gene expression (GE) studies of AA in an independent cohort of 96 unrelated individuals.[16] This work identified a signature composed of 3445 genes differentially expressed between patients with AA and healthy controls, and underscored contributions from the pathways identified in our genetic studies.[6,16] While GE studies are the standard experimental approach for detecting effects of changes in transcriptional levels on disease, these studies tend to identify hundreds or thousands of genes that are differentially expressed between diseased and healthy tissue without providing information about causal order. In GE analysis, genes that are a primary *cause* of disease are not readily distinguishable from genes whose transcript levels are altered secondarily, as a *consequence* of disease. This distinction becomes critical when developing strategies for therapeutic targeting, which ideally should be directed towards genes that are causal rather than consequential.

While many observed transcriptional changes in tissues result from cellular responses to microenvironmental cues (eg developmental, homeostatic or pathogenic cellular signalling), GE changes can also result from underlying structural alterations in the genome that affect gene dosage. Copy number variants (CNVs) are genetic variants that involve the duplication or deletion of segments of the genome greater than one kilobase (kb) and represent an important source of variation in the human genome.[17,18] CNVs, similar to single nucleotide variants, span the full range of allele frequencies, including de novo, rare or common ($f > 1\%$).

Previous work has established that both rare and common CNVs can influence the development of disease. Rare or de novo CNVs have been studied within the context of rare congenital diseases and syndromes. The rare CNVs identified in these studies tend to be large (usually >100 000 base pairs) and often disrupt multiple protein-coding genes and regulatory elements. While CNVs are present in every human genome, burden analyses in cohorts of congenital disorders have shown that affected patients are more likely to harbour large gene-disrupting (genic) CNVs than unaffected individuals.[19–22] Likewise, common CNVs have been identified in association studies of autoimmune diseases such as Crohn's disease,[23–25] systemic lupus erythematosus (SLE),[26] psoriasis,[27] rheumatoid arthritis (RA), type 1 diabetes,[25,28] and AA.[29]

Copy number variants are likely to influence disease through transcriptional dosage effects. Gene transcript levels may be reduced or absent when a gene lies within a deletion CNV or amplified within a duplication CNV.[30–35] Thus, we postulate that a gene whose altered gene expression levels contribute to AA has the potential to be identified in both GE data (comparing diseased tissue to healthy tissue) and CNV analysis (comparing a cohort of unrelated patients and healthy controls). Furthermore, whereas GE studies are cross-sectional in nature, identifying transcriptional changes that occur both before and *after* disease onset, CNV studies, in contrast, may help to identify genetic variants that are present *before* disease and thus tend to be causal rather than consequential. Finally, we postulate that large CNVs exerting strong effects on gene expression levels will have a negative impact on health and are therefore likely to be under purifying selection, remaining at low frequencies in the population.

The goal of this study was to identify large, rare CNVs that are present in the genomes of patients with AA and absent from unaffected controls, and then integrate the CNV and GE data to identify genes that are potential drivers of AA pathogenesis. We identified large, rare gene-disrupting CNVs in a cohort of 758 AA cases that were not included in our GE studies and compared these with CNVs found in 17 769 publicly available unrelated controls.[36,37] We first used these data to evaluate the burden of CNVs between cases and controls and found that patients with AA harbour more of these CNVs than healthy controls (log-rank test, $P = 7.8 \times 10^{-7}$). We next integrated our CNV results with AA GE data and identified 14 genes impacted by large, rare CNVs in patients with AA and whose transcript levels are altered in AA skin in a consistent direction (gain or loss of copy number and up- or down-regulated expression levels, respectively). Four of the 14 genes were impacted by CNVs in three or more patients, including genes that have previously been implicated in hair follicle biology.

2 | MATERIALS AND METHODS

2.1 | Subjects

Patients with AA were ascertained through the National Alopecia Areata Registry with approval from institutional review boards of the contributing sites and genotyped on the Illumina HumanHap 610-Quadv1 or HumanHap 550 chip for our previous GWAS.[4] Controls consisted of individuals that were genotyped on HumanHap550, Illumina HumanHap 610-Quadv1, Illumina HumanHap 1M, Illumina HumanHap iM duo chips for 11 genome-wide association case-control or longitudinal studies unrelated to AA, as described previously.[21,22,38,39] Ancestry-specific SNP genotypes called from the arrays were used in principal component analysis to determine genetic ancestry of each case and control. Only genetically European samples were used in our CNV analysis. Relatedness was also assessed with SNP genotypes to insure that cases and controls were unrelated.

2.2 | CNV calling and quality control assessment

The same analytic pipeline was used for quality control and CNV calling in cases and controls. The CNV calls were generated by PennCNV (version 2011 June).[40] To prepare the input files for PennCNV, the raw intensity data for the genotypes of patients with AA were first normalized using Illumina's Genome Studio software. To generate the raw CNV calls, we used the log R ratio (LRR), B allele frequency (BAF), automatically computed from the signal intensity files, and the standard hg18 "all" PennCNV hidden Markov model (hmm) and population frequency of B allele (pfb) files. High-quality CNVs (confidence score ≥ 30; logR ratio SD ≤ 0.35) were called in 758 cases and 17 769 controls. We limited our analysis to CNVs on autosomes that: (a) fell within or spanned one or more genes, and (b) were within a size range of 100 000 base pairs (bp) and 10 Mb.[41]

2.3 | CNV validation with quantitative PCR

We performed quantitative PCR (qPCR) to validate the selected CNVs. Primers were designed to target the specific genes contained within the CNVs and qPCR was performed using Power SYBR® Green Master Mix on ABI PRISM 7300 Real-Time PCR System (Applied Biosystems). All samples were run in triplicate, and the data were established using the relative standard curve method. The data were normalized against the reference gene, GAPDH, and the copy number was determined empirically by calculating the expression fold change relative to the normal control reference DNA, which does not carry the specific CNV. The relative copy number values ≥1.19 and ≤0.81 were representative of duplications and deletions, respectively.

2.4 | CNV integration with GE data

Alopecia areata gene expression signatures were previously identified by comparison of whole scalp skin tissue samples between patients and unrelated unaffected controls. Specifically, three different comparisons generated three signatures and included a comparison of controls to: (a) all patients with AA, (b) patients with mild disease (transient or patchy AA), or (c) patients with severe disease (universalis or totalis). For our analysis, we combined the three signatures to identify a set of 3658 probes differentially expressed in at least one comparison, representing 3445 genes.

2.5 | CNV burden analysis

Copy number variant burden analysis was performed by counting large (size greater than or equal to 100 kb), rare (frequency <0.1% in the control data set), autosomal CNVs intersecting or containing at least one exonic sequence. CNVs larger than 10 Mb were excluded from the burden analysis due to their relative infrequency and the limited sample size for cases. To examine the global CNV burden, the population frequency of the largest CNV per genome was compared in AA cases and normal controls (Figure 1). The population frequencies of the largest CNVs per genome were analysed using a log-rank test, Wilcoxon test and Tarone-Ware test (SPSS IBM v.24). *P* values of less than 0.05 were considered significant for the above analyses.

2.6 | Immunofluorescence analysis

Snap-frozen human hair follicle sections were postfixed using 4% (wt/vol) paraformaldehyde fixation for 10 minutes at room temperature. Samples were washed twice in PBS for 5 minutes and permeabilized for 10 minutes using PBS with 0.2% Triton X-100. Samples were then washed again in PBS for 5 minutes. Blocking was performed for 20 minutes using 5% (wt/vol) goat serum in PBS with 0.1% Triton X-100. The following primary antibodies were used: rabbit anti-ATG4B (1:500; Invitrogen) and rabbit anti-SMARCA2 (1:100, Abcam). Primary antibodies were incubated in blocking solution at RT for 1 hour. Primary antibodies were washed off using two 15-minute washes in PBS with 0.1% Triton X-100 and then three consecutive 5-minute PBS

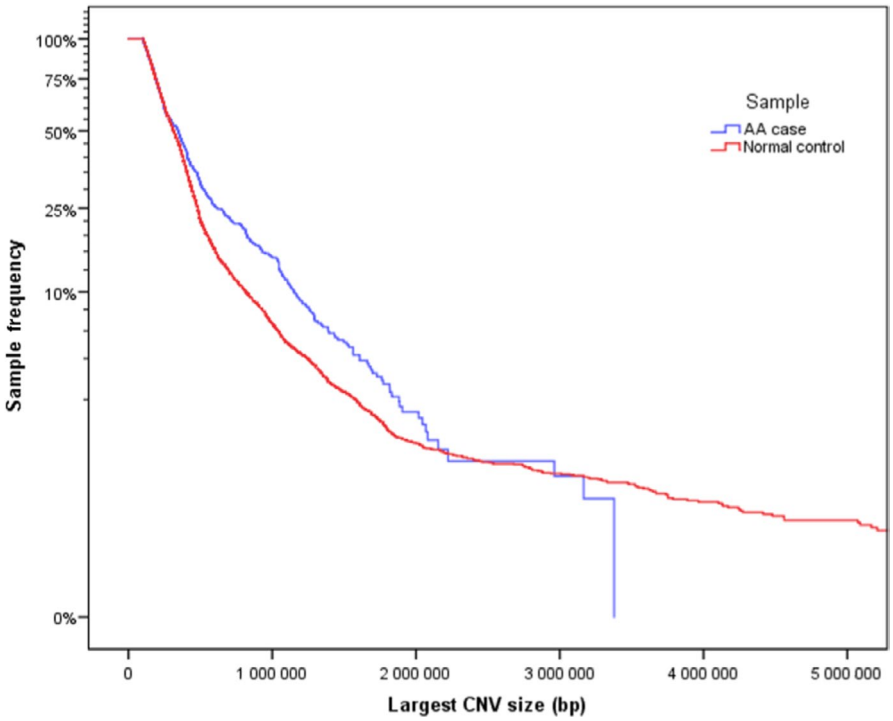

FIGURE 1 Alopecia areata (AA) cases have an increased burden of copy number variants (CNVs). Kaplan-Meier curve showing autosomal CNVs >100 Kb and <10 Mb (N = 2410) for 673 cases vs 16 311 controls (log-rank test, $P = 7.8 \times 10^{-7}$). Our data suggest that there may be a slight enrichment of CNVs greater than 500Kb in size among patients with AA relative to controls. Estimates for CNVs >1.5 Mb in cases may be imprecise due to the small number of observations in our sample

washes. Secondary antibody (Alexa Fluor 488 goat anti-rabbit 1:1000; Invitrogen) was applied for 30 minutes at room temperature. Samples were washed using two 15-minute washes in PBS with 0.1% Triton X-100 and then one more PBS wash. Samples were mounted using Fluoroshield with DAPI (Sigma), and slides were visualized on a Zeiss Exciter confocal microscope.

This work is approved by Columbia University IRBs AAAA8075 and AAAI0706.

3 | RESULTS

3.1 | CNV characterization

Of the 758 cases and 17 769 controls for which CNVs were called, we identified 673 cases and 16 311 controls that contained at least one CNV meeting our inclusion criteria, which included a low frequency in controls ($f < 0.1\%$) and a size range of 100 kb to 10 Mb. Among the 673 AA cases, we identified 2888 genes affected by CNVs, 2506 of which were also affected by a CNV in at least one control. However, when we removed CNVs that were present in both cases and controls to identify CNVs only ever observed among our cases, we identified 382 genes that were uniquely impacted by CNVs in AA cases.

3.2 | CNV validation with quantitative PCR

We performed genomic qPCR to experimentally validate a sample of CNVs harboured by patients with AA (Table S1). We validated 47 (92.2%) of 51 CNVs using qPCR. Two of the four CNVs that failed validation were <18 000 bp, and the relative lower confidence associated with calling such small CNVs likely generated the unreliable CNV calls.

3.3 | CNV burden analysis

Previous studies of rare, large CNVs found that they significantly contribute to disease burden.[22] To evaluate whether patients with AA carry a greater number of CNVs than controls, we conducted a burden analysis by selecting the largest CNV in each individual and examining the distribution of CNVs in AA cases and controls. We observed an excess burden of rare, large, gene-disrupting CNVs in AA cases compared with controls (log-rank test, $P = 7.8 \times 10^{-7}$; Wilcoxon, $P = 3.6 \times 10^{-3}$; Tarone-Ware, $P = 4.4 \times 10^{-5}$). Our data suggest that patients with AA tend to carry more CNVs within a discrete size range, specifically between 500 Kb and 1.5 Mb in size ($P = 4.4 \times 10^{-5}$, Tarone-Ware test; Figure 1). We did not observe enrichment in patients with AA for CNVs greater than 1.5 Mb

3.4 | CNV integration with gene expression data

We identified 35 genes (out of 382 genes affected by CNVs in AA cases and never in controls) that were present in at least one of the previously published AA GE signatures (all AA, severe disease, mild disease). Of these 35 genes, only 14 had a consistent direction of effect within the CNV across all experiments (Table 1). Specifically, seven

TABLE 1 Genes identified by copy number variant (CNV) analysis as potential contributors to alopecia areata pathogenesis

						CNV patient count		Gene expression	
	Region	Gene	chr	Hg38.Start	Hg38.Stop	CNV deletion	CNV duplication	Fold change	Signature
Decreased expression	2q37.3	BOK	2	241 558 721	241 574 138	4		−1.58	AU
		ATG4B	2	241 637 213	241 673 857	6		−1.59	AAP,AA,AU
	3q25.1	TM4SF1	3	149 369 022	149 377 865	1		−3.19	AAP,AA,AU
	3q29	NCBP2	3	196 935 402	196 942 597	1		−1.65	AU
	9p24.3	SMARCA2	9	1 980 290	2 193 624	3		−1.56	AA,AU
	19q13.43	ZNF814	19	57 848 731	57 889 074	4		−1.86	AA,AU
		ZNF274	19	58 183 029	58 213 562	1		−1.59	AU
Increased expression	9q33.3	RALGPS1	9	126 914 774	127 223 166		1	1.8	AU
	10p12.1	YME1L1	10	27 110 112	27 155 266		1	2.34	AA,AU
	19p13.3	ZNF57	19	2 900 898	2 918 476		1	1.55	AU
	19p13.3	CELF5	19	3 224 703	3 297 076		1	1.69	AU
	1p36.22	CTNNBIP1	1	9 848 276	9 910 336		1	1.77	AU
	2q11.1	FAHD2A	2	95 402 721	95 416 616		1	2.06	AA,AU
	2q37.1	SP140	2	230 203 110	230 313 215		1	1.96	AA,AU

genes were down-regulated in the AA GE signature and were impacted by a CNV deletion (copy number state = 1); and seven genes were up-regulated in the AA GE signature and were impacted by a CNV duplication (copy number state = 3; Table 1). The majority of these genes fell within a CNV observed only once in our sample of AA cases. Four of these genes are affected by CNVs in three or more patients with AA. The cohort of patients with AA used for CNV analysis is independent of the cohort of patients with AA used for GE analysis.

Notably, deletions in three genomic regions (affecting a total of four genes) were identified in multiple AA patients, including ATG4B/BOK (chromosome 2q37.3), SMARCA2 (chromosome 9p24.3) and ZNF814 (chromosome 19q13.43). ATG4B and BOK are located in the same genomic region on chromosome 2 (Figure 2). One CNV spans both genes in four patients. Two additional patients have a CNV that spans ATG4B but does not affect BOK. On chromosome 9, SMARCA2 is the only gene affected by CNVs in that region of the genome. On chromosome 19, one patient carries a deletion affecting both ZNF274 and ZNF814, and three patients have a CNV spanning ZNF814, but not ZNF274.

3.5 | Protein expression in hair follicle

Immunofluorescence staining of ATG4B and SMARCA2 in healthy human anagen hair follicles revealed distinct expression patterns. ATG4B (Figure 2A) showed perinuclear expression, with stronger staining in the hair matrix that decreases in the hair shaft cortex (HSCx). The dermal papilla showed positive yet diffuse ATG4B staining. SMARCA2 (Figure 2B) showed widespread nuclear protein localization throughout the hair follicle in the outer root sheath (ORS), matrix and dermal papilla, with an overall weaker expression in the dermal sheath and HSCx.

4 | DISCUSSION

The genotype data generated in our GWAS together with access to a large sample of control genotype data provided an opportunity to investigate an aetiological role for CNVs in AA. Our previous linkage studies in AA families provided evidence that rare genetic variation contributes disease pathogenesis, thus providing a rationale to pursue the investigation of rare CNVs. Furthermore, recent sequencing efforts in common diseases have shown that the genetic architecture of common diseases includes variants across the whole spectrum of population frequencies, including rare variants.[42–45] Importantly, rare variants can provide clinically relevant mechanistic insight that can be extrapolated to a majority of patients.[13,15,46–50]

While our cohort size could limit the statistical power to detect rare variants and increase type I error, our integrative analytic strategy mitigates the chance of false positives. Specifically, we postulated that if a gene influenced AA through altered transcript levels, it would be identified in both CNV data and GE data generated from independent cohorts. We further increased our experimental stringency by restricting our analysis to CNVs observed *only* in our AA cases, and *never* in our population controls. This integrative approach enabled us to overcome the main limitation of GE approaches, which are cross-sectional in nature and fail to distinguish causes from consequences of disease.

Our burden analysis indicated that patients with AA are more likely to carry large rare CNVs (ranging from 100 kb to 10 Mb) than population controls (Figure 1). However, it remains unknown if or how many patients with AA in our cohort have congenital disorders that include AA as a symptom or co-presenting disorder. For example, trisomy 21 is known to increase the risk of AA, and we identified three patients with genetic evidence of trisomy 21.[51]

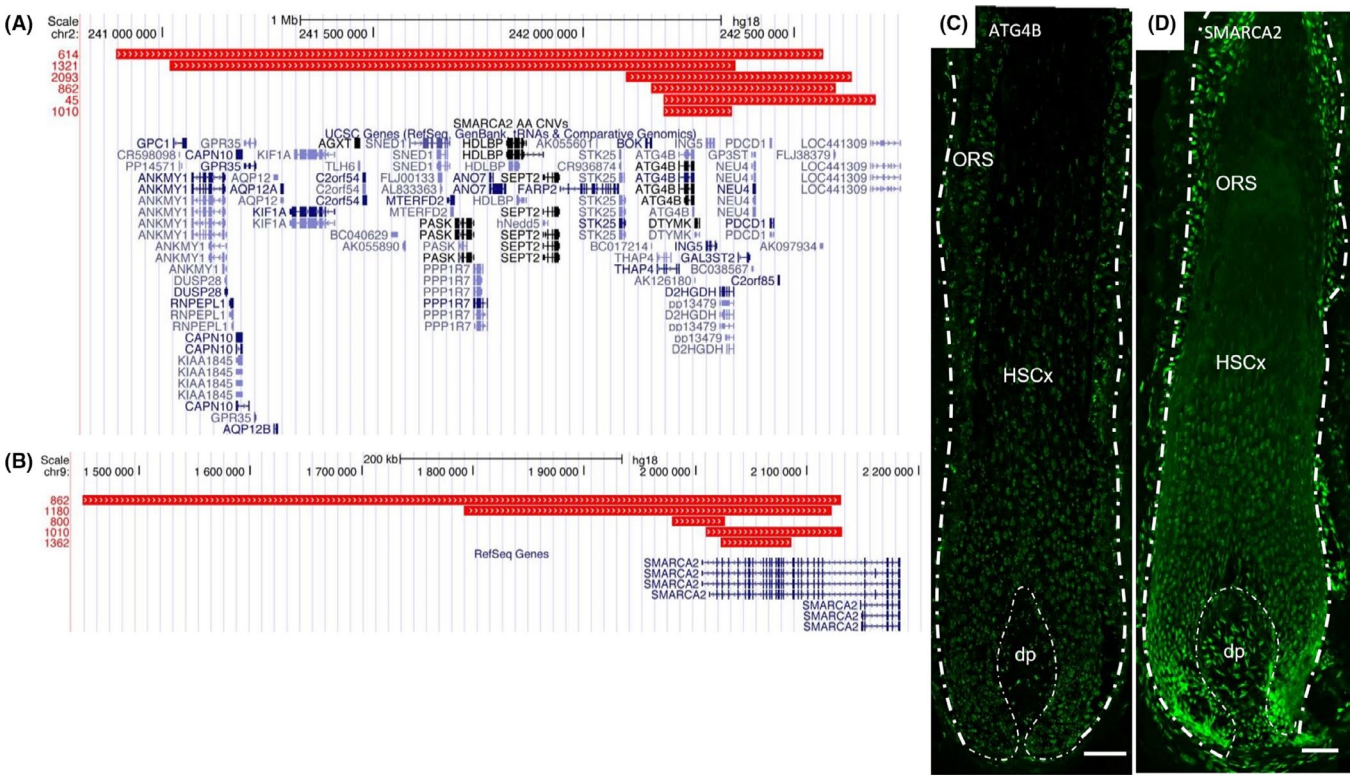

FIGURE 2 Genes impacted by copy number variants (CNVs) in multiple alopecia areata (AA) patients. A, Genomic display of CNV deletions observed in patients with AA is shown in red across the ATG4B genomic region. While all of the CNVs span five genes, only ATG4B is differentially expressed in AA lesional skin.[16] B, Genomic display of CNV deletions observed in patients with AA are shown as red bars across the SMARCA2 genomic region. C, ATG4B immunofluorescence staining of a healthy human anagen hair follicle reveals perinuclear expression in hair follicle keratinocytes. The dermal papilla shows positive yet diffuse ATG4B staining, with stronger staining in the hair matrix that tapers off in the hair shaft cortex (HSCx). D, SMARCA2 staining reveals nuclear protein localization in the human hair follicle. Expression appears throughout the hair follicle in the outer root sheath (ORS), matrix and dermal papilla with weaker expression in the HSCx and dermal sheath

Thus, it remains unknown if the increased burden that we detected is generalizable to all patients with AA. Among patients with AA, we did not detect any CNVs >1.5 Mb in size. Larger CNVs tend to disrupt more genes and therefore are more likely to have severe health consequences and remain at low population frequencies. Given the relatively small number of cases included in our study, we may have limited power to detect such rare events of CNVs > 1.5 Mb.

While our analysis identified 14 genes residing in 12 genomic regions, only four genes were affected by CNVs found in more than one patient, thereby increasing our confidence in potentially causal disease effects. While none of these genes have previously been implicated in AA by our GWAS or linkage studies, two of them (ATG4B and SMARCA2) have been previously implicated in hair follicle biology.

ATG4B encodes a cysteine protease and is a core autophagy protein that is located on chromosome 2.[52,53] Autophagy is an intracellular mechanism for capturing cytosolic proteins or organelles for transport to the lysosome for degradation or to the plasma membrane for cellular export. ATG4B has been implicated in a wide range of diseases, including cancer, pathogen clearance deficiencies and other autoimmune diseases.[54,55] Atg4b-null mice present with autophagy deficits, differential expression of proinflammatory cytokines implicated in bacterial innate immune responses and an increased risk for induced colitis.[56–58] This mouse model mimics multiple symptoms present in Crohn's disease and ulcerative colitis, and has been proposed as a novel autophagy-deficient model for testing new therapeutics in IBD and colitis.[58] In humans, Crohn's disease GWAS identified the ATG4B locus as a susceptibility gene (rs35320439, $P = 9.89 \times 10^{-10}$, OR = 1.09),[59,60] and reduced expression of ATG4B was found in the inflamed colon region of patients with IBD.[58] ATG4B has also been implicated in prostate cancer by GWAS (rs3771570, $P = 5 \times 10^{-9}$, OR = 1.12)[61] and in chronic myeloid leukaemia (CML) where it has been proposed as a potential biomarker for predicting therapeutic response in treatment-naïve CML stem/progenitor cells.[62]

Several lines of evidence implicate ATG4B in hair follicle biology. In a growth hormone-deficient transgenic rat model for hair follicle regeneration, ATG4B was markedly up-regulated after depilation of telogen-phase hairs.[63]In melanocytes, ATG4B plays a critical role in melanosome trafficking, and earlier studies in primary melanocytes demonstrated that induction of autophagy can increase pigmentation while autophagy inhibition reduces pigmentation.[64]

Recently, ATG4B knockdown was found to disrupt melanosome trafficking within the cell, resulting in the perinuclear accumulation of the melanosomal organelles.[64] ATG4B knockdown also reduced the co-localization of premelanosome protein (PMEL) with LC3, a core autophagy protein.[64] In our study, we found that ATG4B exhibits perinuclear expression with strong staining in the hair matrix of human hair follicles.

Taken together, the genetic evidence in other autoimmune diseases, along with functional evidence in hair follicle and melanocyte biology, suggests a plausible role for ATG4B in AA disease pathogenesis. Of note, autophagy has been implicated in AA by our previous GWAS and meta-analysis, specifically by associations with STX17 ($P = 1.09 \times 10^{-5}$) and BCL2L11, also known as BIM ($P = 1.5 \times 10^{-8}$), both of which have known roles in autophagy pathways, as well as PMEL ($P = 4.4 \times 10^{-9}$).[4,5,65] The precise mechanisms by which autophagy contributes to AA pathogenesis have yet to be fully elucidated.

In four of the six AA patients with an ATG4B CNV deletion, the CNV also encompasses a neighbouring gene, BOK, which is located approximately 100 kb away. Similar to ATG4B, BOK is also downregulated in lesional skin of patients with AA. BOK is a pro-apoptotic member of the BCL2 family that also induces autophagy.[66,67] Therefore, it is possible that in patients with AA the tandem deletion of ATG4B and BOK could result in an additive effect in which multiple autophagy genes are perturbed, causing a more pronounced deficit in the pathway. Future studies are needed to further refine contributions from BOK.

Our analysis also identified SMARCA2 (also known as Brahma, BRM)[68] located on chromosome 9 as a potential contributor to AA pathogenesis. This gene encodes a transcriptional activator protein that functions as a subunit of the Switch/Sucrose non-fermentable (SWI/SNF) complex.[69,70] The SWI/SNF complex is crucial in regulating cellular growth, differentiation and division through chromatin remodelling.[71] SMARCA2 and other SWI/SNF subunits have essential roles in tumor suppression and are implicated in many types of cancer when dysregulated.[72–74] Furthermore, SMARCA2 has been proposed as a potential therapeutic candidate for targeting specific cancers with Brahma-related gene-1 (BRG1) mutations, such as non–small-cell lung carcinoma, Burkitt's lymphoma and childhood medulloblastoma.[74,75]

SMARCA2 mutations were previously associated with diseases characterized by hair phenotypes.[76–78] For example, SMARCA2 mutations have been identified in Nicolaides-Baraitser syndrome (NBS), which manifests with hypotrichosis.[77,78] Substantial phenotypic overlap exists with NBS and another rare genetic disease, Coffin-Siris syndrome (CSS), a condition that exhibits hypotrichosis, as well as facial hypertrichosis.[76,77] SMARCA2 sequencing in 44 patients with NBS revealed causative missense mutations in ~80% of patients, and functional analysis suggested a dominant-negative effective of these mutations.[78,79] In a CNV analysis, four CSS patients with were identified to have a chromosome 9p duplication encompassing 43 protein-coding genes, one of which was SMARCA2.[76] It was postulated that the increase in SMARCA2 gene dosage was contributing to the CSS phenotype.[76] The implication of SMARCA2 in cancer and hair-related conditions provide a rationale for the plausible involvement of SMARCA2 in AA pathogenesis. Here, we present immunofluorescent staining that demonstrates expression of SMARCA2 throughout the human hair follicle, including the dermal papilla, outer root sheath (ORS) and matrix.

Zinc finger protein 814 (ZNF814) on chromosome 19 has not previously been studied within the context of skin or hair biology. It has been reported to be differentially expressed in parous vs nulliparous breast tissue and may play a role in pancreatic cancer and squamous cell carcinoma.[80–85] In the four AA patients with a CNV deletion of ZNF814, the neighbouring gene, ZNF274, was also deleted in one patient. ZNF274 is also a zinc finger protein that acts as a transcriptional factor and complexes with chromatin-remodeler ATRX (alpha thalassaemia/mental retardation syndrome X-linked), a SWI/SNF-family protein.[86] Depletion of ZNF274 results in cell cycle dysfunction, DNA damage and decreased H3K9 trimethylation.[86]

Our work demonstrates that rare CNVs can be leveraged to identify and prioritize drivers of disease within GE datasets in patients with AA. These findings provide further evidence for aetiological contributions from rare variants in AA in support of our previous linkage analysis. Our integrative analysis of CNV and GE data uncovered evidence that implicated at least two new genes in AA (ATG4B and SMARCA2), suggesting a role for dysregulated autophagy in AA pathogenesis.

ACKNOWLEDGEMENTS

We thank the patients and their family members who participated in this study. We are grateful to David Norris, Maria Hordinsky, Madeleine Duvic, Julian Mackay-Wiggan and Vera Price. This work was supported in part by NIH/NIAMS P50AR070588 Alopecia Areata Center for Research Translation (AACORT), NIH/NIAMS R01AR065963 Functional Genomics of Alopecia Areata and NIH/NIAMS U01AR067173 Developing an Alopecia Areata Disease Activity Index (ALADIN), R01AR52579 Genetic Analysis of Alopecia Areata and R01AR056016 Genome-wide Association Studies in Alopecia Areata. The patient cohort was collected and maintained by the National Alopecia Areata Registry (N01AR62279). We are grateful for the support of the National Alopecia Areata Foundation for funding initial studies.

CONFLICT OF INTEREST

The authors have declared no conflicting interests.

AUTHOR CONTRIBUTIONS

LP contributed to the project conceptualization, data curation, formal analysis, investigation, methodology, visualization and writing. AVP contributed to the data curation, investigation, validation and visualization. RKR contributed to the data curation, formal analysis, investigation, validation and visualization. LB contributed to

the formal analysis, investigation and visualization. MV contributed to the data curation, formal analysis and investigation. SSC contributed to the data curation, formal analysis, investigation, methodology and validation. SE and AA contributed to the investigation, validation, visualization and writing. JEC contributed to the data curation and investigation. AJ contributed to the data curation, formal analysis and investigation. AMC contributed to the conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation and writing.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Report of all CNVs found in AA patients and not in controls

How to cite this article: Petukhova L, Patel AV, Rigo RK, et al. Integrative analysis of rare copy number variants and gene expression data in alopecia areata implicates an aetiological role for autophagy. *Exp Dermatol.* 2019;00:1–11. https://doi.org/10.1111/exd.13986