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Genome-wide analysis of copy number variants in alopecia areata in a Central European cohort reveals association with *MCHR2*

Running Head: Copy number variants in alopecia areata

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Abstract

Alopecia areata (AA) is a common hair loss disorder of autoimmune etiology, which often results in pronounced psychological distress. Understanding of the pathophysiology of AA is increasing, due in part to recent genetic findings implicating common variants at several genetic loci. To date, no study has investigated the contribution of copy number variants (CNVs) to AA, a prominent class of genomic variants involved in other autoimmune disorders. Here, we report a genome-wide- and a candidate gene focused CNV analysis performed in a cohort of 585 AA patients and 1,340 controls of Central European origin. A nominally significant association with AA was found for CNVs in the following five chromosomal regions: 4q35.2, 6q16.3, 9p23, 16p12.1, and 20p12.1. The most promising finding was a 342.5 kb associated region in 6q16.3 (duplications in 4/585 patients; 0/1,340 controls). The duplications spanned the genes *MCHR2* and *MCHR2-AS1*, implicated in melanin-concentrating hormone (MCH) signaling. These genes have not been implicated in previous studies of AA pathogenesis. However, previous research has shown that *MCHR2* affects the scale color of barfin flounder fish via the induction of melanin aggregation. AA preferentially affects pigmented hairs, and the hair of AA patients frequently shows a change in color when it regrows following an acute episode of AA. This might indicate a relationship between AA, pigmentation, and MCH signaling. In conclusion, the present results provide suggestive evidence for the involvement of duplications in *MCHR2* in AA pathogenesis.

Key words

alopecia areata, copy number variants, molecular genetics, genetic complex disorder, MCHR2

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Introduction

Alopecia areata (AA) is a common hair loss disorder, and one of the most prevalent autoimmune-mediated disorders in humans. The estimated lifetime risk is around 1.7 - 2 % (1, 2). Similar rates are observed in both sexes, and no evidence exists for a peak incidence in any specific age group (1, 2). Episodes of hair loss typically commence with the development of non-scarring, isolated hairless patches. These then extend centrifugally and may coalesce. AA episodes are characterized by both a substantial increase in the proportion of telogen hairs, and the development of dystrophic hairs (exclamation mark hairs). Although the scalp is the predominantly affected site, all hair bearing areas of the body can become involved. AA is often characterized by a sudden onset and a variable clinical course. In many affected individuals, the stigmatizing potential and unpredictable course of the disorder result in pronounced psychological distress. Currently available therapies are suboptimal, and most are only effective in mild cases, and thus new treatment strategies are warranted. The development of novel treatments for AA will require a more comprehensive understanding of pathophysiology. Research suggests that the main underlying pathomechanism of AA is a T-cell mediated, tissue-specific autoimmune response. However, understanding of this process remains limited.

Consensus exists among researchers that genetic factors play an important role in the development of AA, and that their identification will elucidate the underlying biological processes (3-5). Recent genome-wide association studies (GWAS), and focused candidate gene studies have identified a total of 12 susceptibility loci for AA. These include loci in the HLA region, with HLA-DR being the probable key etiological factor (6), and nine other loci of relevance to autoimmunity (6-8). In addition to common variants, rare genetic variants may also play an important role in the development of AA. As is the case with common variants, associations with rare loci

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may point to important steps in AA development. Furthermore, since effect sizes for rare variants are typically larger than for common variants, the former may offer advantages in terms of functional follow-up studies. The availability of array-based genome-wide single nucleotide polymorphism (SNP) data allows the efficient characterization of copy number variants (CNVs). These are a prominent class of genetic variants affecting larger genomic regions, which have been implicated in a number of autoimmune disorders, such as rheumatoid arthritis (9), Crohn's disease (10, 11), systemic lupus erythematosus (12, 13), psoriasis (14-16), and atopic dermatitis (17). To our knowledge, no genome-wide CNV-analysis of AA has been performed to date.

The aim of the present study was to analyze the relevance of CNVs in AA by investigating 585 AA patients and 1,340 population based controls of Central European descent. Two separate analyses were performed: first, an initial genome-wide CNV analysis, and second, a subsequent focused analysis of candidate genes previously implicated in AA pathogenesis.

Materials and Methods

Sample description

The study protocol was approved by the respective ethics committees, and complied with the Declaration of Helsinki. All participants provided written informed consent prior to inclusion.

Patient cohort

Male and female patients were recruited from the following sources: outpatient clinics in Belgium and Germany, private dermatology practices in Germany, and AA self-help support groups in Germany and the Netherlands. The inclusion criterion was a

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dermatologist-assigned diagnosis of AA. All participants were of Central European descent according to self-reported ancestry. Patients with Down syndrome or Turner syndrome were excluded.

After quality control (QC) 585 patients remained in the dataset. For these participants, the mean age of onset was 29.1 years (standard deviation (SD) = 17.2). Patchy AA was defined as one or more circumscribed patches of hair loss. Alopecia totalis (AT) was defined as 100% loss of scalp hair without loss of body hair. AT/AU was defined as 100% loss of scalp hair with variable loss of body hair. Alopecia universalis (AU) was defined as 100% loss of both scalp and body hair. The majority of patients (68.5%) had patchy AA. The remaining patients were classified as having: AT (7.5%); AU (9.6%); or AT/AU (14.4%).

Control cohort

Population-based controls were drawn from the following three epidemiological studies: KORA (Cooperative Health Research in the Region of Augsburg, Germany) (18), PopGen (Population-Based Recruitment for Genetics Research) (19), and HNR (Heinz Nixdorf RECALL [Risk factors, Evaluation of coronary calcium and lifestyle]) (20). After QC, 1,340 controls remained in the study.

Genotyping and quality control prior to CNV detection

Patients were genotyped on Illumina's Human660W-Quad microarray (Illumina Inc., San Diego, CA, USA). Controls were genotyped on Illumina's HumanHap550v3 microarray (Illumina Inc., San Diego, CA, USA). Only markers that were common to both array types were analyzed (n = 546,356 SNPs). To ensure high data quality and to avoid technical artifacts, the following stringent QC criteria were applied, as described elsewhere (21): SNPs with a call rate of < 97% and individuals with a DNA

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call rate of < 97% were removed from the analysis, individuals with a difference between X-chromosomally inferred and phenotypic sex were excluded and DNA sample duplicates identified by identity by state (IBS) estimates (IBS = 2) were removed as well as cryptic related individuals (IBS ≥ 1.6) and population outliers, as defined according to multi-dimensional scaling with HapMap phase 2 data.

CNV detection and quality control

Putative CNVs were identified using QuantiSNP (QS, version 2.1, http://www.well.ox.ac.uk/QuantiSNP) (22), and PennCNV (PC, 2011June16 version; http://www.openbioinformatics.org/penncnv/) (23). Neither the X-chromosome nor the MHC region was analyzed. Individuals were excluded if their SD from the log R ratio exceeded 0.30, as calculated over all SNPs. Only those CNVs that were detected by both QS and PC were included in the genome-wide- and the candidate gene analysis.

Genome-wide CNV analysis

All CNVs were required to span at least 30 SNPs, and have a log Bayes Factor (lBF; QS) or a confidence value (PC) of ≥ 30. To be included in further analyses, the associated CNV regions were required to show nominal significance according to the CNV calls from both calling programs. Since the position and extent of genomic rearrangements in CNV regions may vary between individuals, the associated regions were required to share at least three consecutive SNPs in all CNV carriers. To allow the inclusion of smaller aberrations in the associated CNV regions, the filter criteria were relaxed (30 > lBF/confidence value > 10; 30 > number of consecutive SNPs > 10).

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Candidate gene CNV analysis

The transcription start and end position of each RefSeq gene was determined using NCBI build 36 and the UCSC Genome Browser (http://genome.ucsc.edu/) (24). All CNVs were required to lie within 20 kb up- or downstream of the boundaries of the assigned RefSeq gene, span at least 10 SNPs, and have a log Bayes Factor (lBF; QS) or a confidence value (PC) of ≥ 10.

Selection of candidate genes

Genome-wide significant SNPs ($p < 5 \times 10^{-8}$) from previous AA studies were selected, and a list of candidate genes was generated (supplementary table 1). This list comprised all genes with an intragenic genome-wide significant SNP, and the nearest upstream and downstream gene for each intergenic SNP. To be included in the study, the candidate gene region (RefSeq gene boundary + 20 kb) required coverage with > 10 SNPs in the genome-wide microarrays.

Statistical analysis of CNVs

The datasets generated by QS and PC were analyzed separately using PLINK (PLINK version 1.07) (25). To test for association between AA and CNVs in specific chromosomal regions/candidate gene regions, Fisher's exact test (two-sided) was used.

Experimental verification of predicted CNVs

All CNVs in the associated regions (genome-wide CNV analysis) and in the candidate genes were visually inspected using Illumina's Genotyping Module 1.9.4 Genome Viewer. In a previous CNV analysis by our group, which involved the presently applied microarray types, all *in silico* CNVs fulfilling the above mentioned

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filter criteria for the genome-wide CNV analysis were verified experimentally (21). As a proof of principle for the present study, we selectively verified all four occurrences of duplications in 6q16.3, which constituted the most promising finding. These duplications were verified experimentally using real-time quantitative PCR and Power SYBR®Green (Life Technologies, Carlsbad, CA). Five primer pairs were used, and their sequences are obtainable upon request. Relative copy numbers were measured in comparison to three housekeeping genes (*BNC1*, *CFTR*, *RPP38*). Since the CNV identified in the present candidate gene CNV analysis spanned more than 30 SNPs, no experimental verification of this CNV was performed.

Results

Genome-wide analysis

The samples of a total of 585 patients and 1,340 controls passed QC and were included in the downstream analyses. For both the QS and the PC dataset, a nominally significant association with the following five chromosomal regions was found (Tab. 1): deletions in a 130.2 kb region on chromosome 4q35.2 ($p = 0.028$), duplications in a 342.5 kb region on chromosome 6q16.3 ($p = 0.008$), deletions and one duplication in a 99.7 kb (duplications and deletions) or 84.8 kb region (deletions only) on chromosome 9p23 ($p = 0.005$ for duplications and deletions; $p = 0.01$ for deletions); deletions and one duplication in a 474.6 kb region on chromosome 16p12.1 ($p = 0.028$), and deletions in a 25.8 kb region on chromosome 20p12.1 ($p = 0.041$). None of these associations withstood correction for multiple testing. Supplementary table 2 shows the chromosomal position and size of each associated CNV detected in the present patient cohort. Individual genotype and intensity data are obtainable upon request. For CNVs whose putative breakpoints differed

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according to PC and QS, predicted CNV sizes common to both programs are reported.

CNVs in 6q16.3

In 6q16.3, four patients carried duplications (0.68%). Using our primary filter criteria, no CNV was detected in the control cohort. The four CNVs had differing putative breakpoints, and their sizes varied between 341.7 kb and 361.1 kb. The associated region spans 342.5 kb (100.40 - 100.75 Mb), and includes the genes melanin-concentrating hormone factor 2 (*MCHR2*) and MCHR2 antisense RNA 1 (*MCHR2-AS1;* **figure 1**). After application of the relaxed filter criteria, a smaller duplication (67.0 kb) was detected within the associated region in one control subject. This duplication affected neither *MCHR2* nor *MCHR2-AS1*, and did not reduce the size of the associated region.

Candidate gene based analysis

A total of 20 genes were selected for the candidate gene analysis. Of these, nine lacked sufficient coverage on the microarrays (< 10 SNPs), and were therefore excluded from the downstream analysis (supplementary table 3).

Within the eleven remaining candidate gene regions, only one CNV was detected. This duplication in 2q13 was found in one patient (0.17 %, $P > 0.05$), and spanned the genes acyl-CoA oxidase-like (*ACOXL*) and BUB1 mitotic checkpoint serine/threonine kinase (*BUB1*). The duplication spans 462.3 kb, and includes 91 SNPs (111.11 - 111.57 Mb). No CNV was detected in the control cohort.

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Discussion

The present study involved a genome-wide- and a candidate gene focused CNV analysis in a cohort of 585 AA patients and 1,340 population-based controls. The application of stringent filter criteria in the genome-wide CNV analysis ensured the selection of larger CNVs, which have a potentially stronger impact than smaller CNVs. This strategy reduces type I errors, whereas type II errors are expected to increase.

Nominal significance was found for CNVs in 4q35.2, 6q16.3, 9p23, 16p12.1, and 20p12.1. The most promising association was observed for a 342.5 kb region in 6q16.3. Here, duplications were detected in four patients but no controls. These duplications spanned the genes *MCHR2* and *MCHR2-AS1*. In the Database of Genomic Variants (DGV; http://dgv.tcag.ca/dgv/app/home) (26) five studies report at least one duplication affecting *MCHR2* (27-30)http://www.1000genomes.org/home). In total, 12 duplications were observed in 57,328 individuals (0.02%). This frequency is similar to that detected in our controls (0%), and therefore supports the present finding of an association between duplications in 6q16.3 and AA (0.68% in our patient cohort).

Although the precise function of *MCHR2-AS*1 remains unclear, *MCHR2* has been characterized in some detail. *MCHR2* is a G-protein coupled receptor for its primary ligand MCH, the melanin-concentrating hormone. Besides its important roles in feeding and energy homeostasis (e.g.(31)), *MCHR2* has been reported to cause lighter scale color in barfin flounder fish via the induction of melanin aggregation (32). Interestingly, AA preferentially affects pigmented hairs, and the hair of AA patients frequently changes to gray or white when it regrows following an acute episode of AA. We speculate that *MCHR2* affecting duplications lead to an increase in gene dosage and might thus enhance, or otherwise interfere with, the function of this gene

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in combination with the additional, synergistic effects of environmental hazards (33). Together, these observations might indicate a relationship between pigmentation, MCH signaling, and AA. Already in previous AA studies, it was theoretically discussed as well as experimentally shown, that melanogenesis-associated autoantigens generated during active hair shaft pigmentation demonstrate a constitutive risk to attract autoreactive CD8+ T cells (34-37). Moreover, in the first GWAS of AA (8), a significant association was found with common variants in *STX17,* a gene associated with the gray hair phenotype in horses (38). The present results provide suggestive evidence for the involvement of a second pigmentation gene in the pathogenesis of AA.

CNVs in a 475 kb region of 16p12.1 were also associated with AA. This region contains the genes *UQCRC2*, *PDZD9*, *C16orf52*, *VWA3A*, *EEF2K*, *POLR3E*, and *CDR2*. The DGV lists several inversions, duplications, and deletions within the associated region. None of the deletions or duplications in the DGV span the entire region of association. To our knowledge, none of these genes has been implicated previously in the pathogenesis of either AA or any other common autoimmune disorder. However, their involvement in the pathogenesis of AA cannot be excluded.

In 20p12.1, deletions located within *MACROD2* were associated with AA. While 11 deletions were detected in our control cohort, no deletion was detected in the patient cohort using our primary filter criteria. Notably, the association signal was lost following application of the relaxed filter criteria, as several smaller CNVs were detected among the patients. This demonstrates the need to be aware of potentially false positive associations arising as a result of the selected filter criteria. The DGV lists numerous deletions affecting *MACROD2*, thus demonstrating that common deletions are part of the allelic spectrum of this gene. We conclude that deletions in *MACROD2* are unlikely to be of relevance to the AA phenotype.

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The CNVs detected in 4q35.2 and 9p23 had no direct effect on any RefSeq gene. However, the possibility that these CNVs are of biological relevance to AA cannot be excluded.

Since the present study represents the first genome-wide CNV analysis of AA, we were unable to compare our results with those of previous reports. Replication analyses of all identified associated CNV regions in independent cohorts are essential in order to evaluate their relevance to the pathogenesis of AA.

No association was detected between AA and CNVs in any of the investigated candidate genes. However, we cannot exclude the possibility that rare deletions and duplications contributing to the allelic spectrum of these candidate genes were overlooked due to a lack of statistical power. It is of note that our focused CNV analysis was restricted to 11 out of 20 AA candidate genes, since the remaining nine genes lacked sufficient coverage on the microarrays. Therefore, studies in larger cohorts, and in cohorts genotyped on more densely covered SNP arrays, are warranted to elucidate the relevance of CNVs in these AA candidate genes.

The present study had several limitations. First, the power of our cohort to detect rare variants with high levels of significance was limited. It is therefore unsurprising that none of the association findings survived correction for multiple testing. Independent replication is necessary to prove or disprove the observed associations. Second, none of the controls were screened for AA. Given that the approximate lifetime risk for AA is 1 - 2%, some of the control CNV carriers may have been affected, and this may have led to false-negative findings for genuinely associated regions. However, mathematical calculations have shown that for diseases with a lifetime prevalence of < 20%, the impact of unscreened versus screened control status is low (39). Third, our stringent filter criteria led to the exclusion of smaller, and potentially genuine, findings. Fourth, we excluded the MHC region from our CNV analysis, as it is prone

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to false positive findings. The largest CNV study on diverse autoimmune disorders to date, which did not include AA, used a purpose-designed array and found that several CNVs in the HLA region were associated with Crohn's disease, rheumatoid arthritis, and type 1 diabetes (11). Therefore, further studies are warranted to investigate the relevance of CNVs in the HLA region to AA.

In summary, the present study represents the first genome-wide CNV investigation of AA, and generated suggestive evidence that duplications in the 6p16 region, which contains *MCHR2,* might be implicated. This supports the hypothesis that genes involved in pigmentation might be of relevance to AA. The present findings require both confirmation in large, independent cohorts, and functional follow-up investigations.

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[1]performed the research, [2]designed the research study, [3]contributed essential reagents or tools, [4]analysed the data, [5]wrote the manuscript

Conflicts of interest disclosure

The authors have no conflicts of interest to declare.

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Table 1 Six associated regions identified in genome-wide CNV analysis

chr	associated region	patients (n=585)	controls (n=1,340)	p-dup+del	OR (CI)-dup+del	p-dup	OR (CI)-dup	p-del	OR (CI)-del	affected gene
4	190,385,789-190,515,967	0 dup 3 del	2 dup 0 del					0.028	- (0.9-∞)	intergenic between *LINC01060* and *LINC01262*
6	100,402,745-100,745,258	4 dup 0 del	0 dup 0 del			0.008	- (1.5-∞)			*MCHR2, MCHR2-AS1*
9	11,810,083-11,909,732[1] 11,820,879-11,905,692[2]	1 dup 7 del	0 dup 5 del	0.005	6.2 (1.5-36.2)			0.011	5.4 (1.2-32.4)	intergenic between *TYRP1* and *PTPRD*
16	21,856,623-22,331,199	1 dup 2 del	0 dup 0 del	0.028	- (0.9-∞)					*UQCRC2, PDZD9, C16orf52, VWA3A, EEF2K, POLR3E, CDR2*
20	14,729,684-14,755,487[3]	0 dup 0 del	0 dup 11 del					0.041	- (0-0.9)	*MACROD2*

Only at least nominally significant P values are displayed. All positions given according to NCBI built 36/hg18. p, P value; OR, odds ratio; CI, 95% confidence interval; dup, duplication; del, deletion. All P values and odds ratios were calculated using Fisher's exact test (two-sided). "-" refers to null observation in one group. [1] associated region for dup + del, associated region is reduced after applying our relaxed filter criteria (11,820,879 – 11,909,732); [2] associated region for del; [3] no association after applying our relaxed filter criteria.

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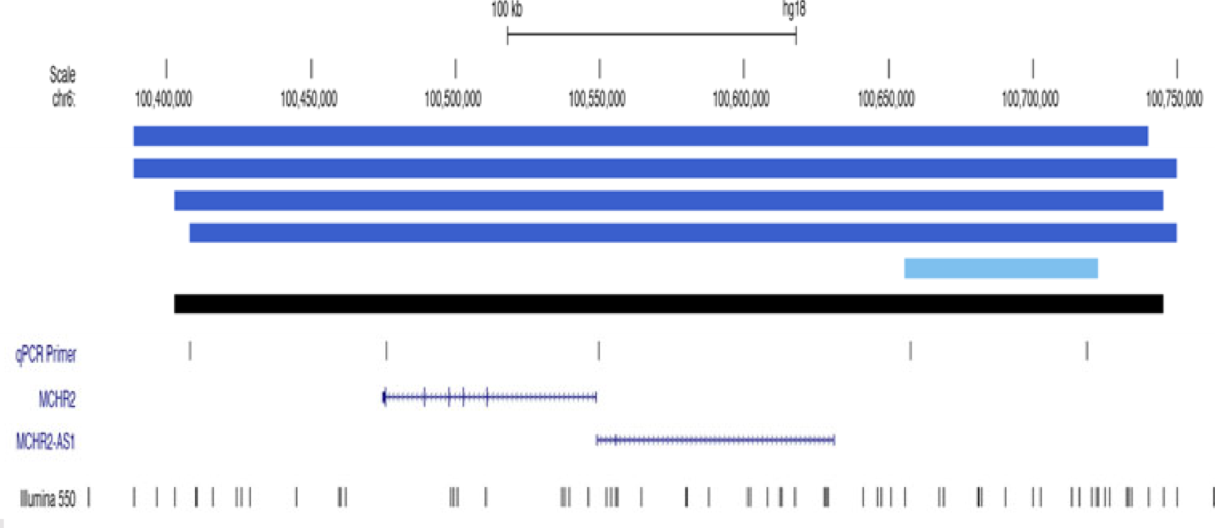
Figure legends

Figure 1

Duplications in 6q16.3

Duplications were detected in four patients (dark blue bars) and in one control (light blue bar). The duplication in the control did not fulfill the primary filter criteria. The associated region (black bar) spanned approximately 342.5 kb. Five primer pairs for SYBR Green verification were designed (black vertical lines). This schematic drawing was generated using UCSC genome browser (http://genome.ucsc.edu/); NCBI36).



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