ORIGINAL ARTICLE

Variant *PADI3* in Central Centrifugal Cicatricial Alopecia

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ABSTRACT

BACKGROUND

Central centrifugal cicatricial alopecia (CCCA) is the most common form of scarring alopecia among women of African ancestry. The disease is occasionally observed to affect women in families in a manner that suggests an autosomal dominant trait and usually manifests clinically after intense hair grooming. We sought to determine whether there exists a genetic basis of CCCA and, if so, what it is.

METHODS

We used exome sequencing in a group of women with alopecia (discovery set), compared the results with those in a public repository, and applied other filtering criteria to identify candidate genes. We then performed direct sequencing to identify disease-associated DNA variations and RNA sequencing, protein modeling, immunofluorescence staining, immunoblotting, and an enzymatic assay to evaluate the consequences of potential etiologic mutations. We used a replication set that consisted of women with CCCA to confirm the data obtained with the discovery set.

RESULTS

In the discovery set, which included 16 patients, we identified one splice site and three heterozygous missense mutations in *PADI3* in 5 patients (31%). (The approximate prevalence of the disease is up to 5.6%.) *PADI3* encodes peptidyl arginine deiminase, type III (PADI3), an enzyme that post-translationally modifies other proteins that are essential to hair-shaft formation. All three CCCA-associated missense mutations in *PADI3* affect highly conserved residues and are predicted to be pathogenic; protein modeling suggests that they result in protein misfolding. These mutations were found to result in reduced PADI3 expression, abnormal intracellular localization of the protein, and decreased enzymatic activity — findings that support their pathogenicity. Immunofluorescence staining showed decreased expression of PADI3 in biopsy samples of scalp skin obtained from patients with CCCA. We then directly sequenced *PADI3* in an additional 42 patients (replication set) and observed genetic variants in 9 of them. A post hoc analysis of the combined data sets showed that the prevalence of *PADI3* mutation was higher among patients with CCCA than in a control cohort of women of African ancestry (P=0.002 by the chi-square test; P=0.006 by Fisher's exact test; and after adjustment for relatedness of persons, P=0.03 and P=0.04, respectively).

CONCLUSIONS

Mutations in *PADI3*, which encodes a protein that is essential to proper hair-shaft formation, were associated with CCCA. (Funded by the Ram Family Foundation and others.)

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Central centrifugal cicatricial alopecia (CCCA) is the most common type of primary scarring alopecia affecting women of African ancestry.[1,2] It is often but not exclusively triggered by hair-grooming habits, which have been suggested as an explanation for its preponderance among women.[1-3] It was originally described in 1968 as "hot comb alopecia" in a series of black women in the United States who straightened their hair with a hot comb and petrolatum products.[4] Unexplained hair breakage may be an early sign of CCCA. It is then followed by hair thinning, mostly involving the vertex scalp. Hair loss progresses centrifugally. Most patients with CCCA present with a seemingly noninflammatory disease resembling androgenetic alopecia. On histopathological examination, the disorder is characterized by varying degrees of lymphocytic inflammation, follicular degeneration, and fibrosis.[2] Its prevalence is estimated to vary from 2.7 to 5.6% among women of African ancestry,[4-6] with a mean age at presentation of 36 years.[7]

Familial occurrence of CCCA has been documented.[1] Genetic susceptibility to the disease is likely to be inherited in an autosomal dominant manner.[8] Here, we report our efforts to delineate the molecular basis of the disorder.

Methods

Study Design and Population of Patients

We recruited patients with CCCA in Durban, South Africa, from 2013 through 2016 and in Winston-Salem, North Carolina, from 2014 through 2017. All the patients provided written informed consent to participate in this study. The cohort of patients comprised two sets: a discovery set and a replication set. Candidate genes were initially searched for with the use of exome sequencing in the discovery set, and we sequenced the top candidate gene in the replication set.

The study was approved by the institutional review boards in Durban and Winston-Salem. The Tel Aviv Medical Center, in Israel, where the sequencing of exomes and targeted sequencing of *PADI3* were performed, has received approval from an institutional review board for the performance of genetic studies.

We attempted to identify candidate genes with variants inherited in a dominant fashion. First, we used three criteria to identify candidate genes in the discovery set: a minor allele frequency of less than 0.05 in persons of African descent and less than 0.0001 in persons of European descent, predicted pathogenicity, and the presence of the candidate gene variants in multiple patients. If a gene was identified as a strong candidate, we would obtain evidence for its role in the pathogenesis of the disease using functional biologic assays. The identification of variants that were functionally shown to be pathogenic in a gene known to encode a protein that plays a critical role in hair-shaft maturation would provide evidence of its role in CCCA pathogenesis.

Nucleic Acid Extraction

Genomic DNA was extracted with the use of kits that were designed to obtain saliva samples (OG-500, DNA Genotek). Total RNA was extracted from biopsy samples of scalp skin with the use of the RNeasy Fibrous Tissue Mini Kit (Qiagen), according to the manufacturer's instructions.

Exome Sequencing

Exome sequencing in patients in the discovery set was performed by Fulgent Genetics or BGI. Whole-exome capture was carried out by in-solution hybridization with SureSelect Human All Exon, version 4.0 (Agilent), or the Roche NimbleGen Protocol (10GB), followed by massively parallel sequencing (Illumina HiSeq2000 or HiSeq4000) with 100–150-bp paired-end reads. Details regarding the methods and exome performance are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

Expression Studies and Enzymatic Assay

RNA sequencing libraries were prepared with the use of the TruSeq RNA v2 protocol (Illumina), and sequencing was performed on a HiSeq 2500 instrument (Illumina). Protein expression was assessed with the use of immunoblotting and immunofluorescence staining of cells and tissues. Enzymatic activity that had been obtained with the various PADI3 expression constructs was measured with the use of an antibody-based assay (ABAP, ModiQuest Research). More details are provided in the Supplementary Methods section in the Supplementary Appendix.

Mutagenesis

We used a vector containing the reference (nonvariant) *PADI3* sequence[9] to engineer the new mutant constructs. Mutagenesis primers were

designed with the use of the Agilent QuikChange Primer Design tool (www.genomics.agilent.com/primerDesignProgram.jsp) and are described in Table S1 in the Supplementary Appendix. The procedure was performed with the use of the QuikChange II XL site-directed mutagenesis kit (Agilent), according to the manufacturer's instructions.

STATISTICAL ANALYSIS

We used the chi-square test and Fisher's exact test to ascertain differences in frequencies of the *PADI3* mutations between the patients and a control population of women of African ancestry in a post hoc analysis. *PADI3* was directly resequenced in both the discovery and replication sets. We did not control for population stratification. The P value threshold was set at less than 0.05 rather than the usual 2.5×10^{-6} that is used for genomewide association studies because this latter design was deemed to be inappropriate for investigating the cause of CCCA given the hypothesis of genetic heterogeneity and the patient sample size. We performed a post hoc analysis involving patients in both the discovery and replication sets, pooled into a single set of patients.

To ascertain differences in enzymatic activity, we used the unpaired Student's t-test to compare normally distributed variables. Values are reported as means with standard deviations. All P values are two-tailed, and a P value of less than 0.05 was considered to indicate statistical significance.

RESULTS

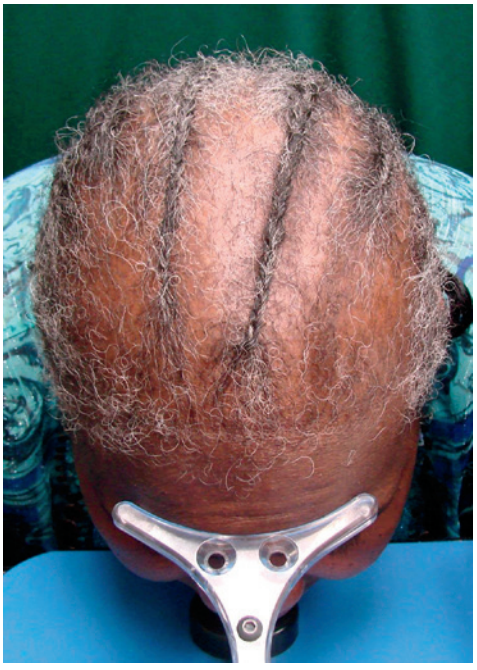
IDENTIFICATION OF CANDIDATE VARIANTS IN PATIENTS WITH CCCA

We initially conducted exome sequencing in a discovery set, which included 16 women of African ancestry who had received a diagnosis of CCCA (Table S2 in the Supplementary Appendix). The discovery set included one familial case (from Family 1) (Fig. S1 in the Supplementary Appendix). Each patient received a clinical diagnosis from a dermatologist at each respective location. For each patient, the diagnosis was confirmed by means of biopsy (Fig. 1). Clinical examination revealed hair loss over the crown, with centrifugal spread and a perifollicular grayish halo on dermoscopy in all patients. All the biopsy specimens showed decreased hair-follicle density and perifollicular lymphocytic infiltration with areas of fibrosis. CCCA was graded in all the patients according to the Central Hair Loss Grading scale (scores range from 0 to 5, with higher scores indicating more severe disease). Patients who were included in the discovery set had a moderate-to-severe condition (score of 3 to 5) (Table S2 in the Supplementary Appendix).[10]

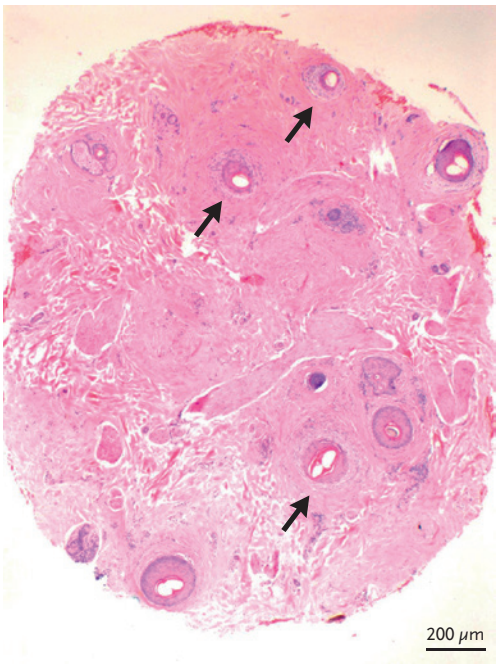
Exome sequencing was performed by Fulgent Genetics in Patients 1 through 10 and by BGI in Patients 11 through 16. Variants that were identified by means of exome sequencing were classified according to their predicted effects on protein function with the use of the PolyPhen-2 (Polymorphism Phenotyping, version 2) tool,[11] Provean (Protein Variation Effect Analyzer) software,[12] the SIFT (Sorting Intolerant from Tolerant) algorithm,[13] and the ConSurf server.[14] The prevalence of CCCA is estimated to be 2.7% among black women in South Africa[5] and 5.6% among black women in the United States.[6] Because CCCA has been reported almost exclusively in women of African ancestry,[4,15,16] we selected for further analysis variants that were predicted to be pathogenic, that were shared by the patients, and that had a minor allele frequency of less than 0.05 in the African population and of less than 10^{-4} in the European population. (Prevalence data were derived from the Genome Aggregation Database [http://gnomad.broadinstitute.org/].)

Using this strategy, we identified four heterozygous mutations in the gene *PADI3* (RefSeq accession number, NM_016233.2) in 5 of 16 patients (31%): c.856A→G, c.1744G→A, c.1669C→T, and c.832-2A→G (Fig. 1C and Table 1). *PADI3* encodes the enzyme peptidyl arginine deiminase, type III. These four mutations included one splice-site mutation and three missense mutations. All the missense mutations had a minor allele frequency in the range of 0.0001 to 0.04 in the African population while being very rare among persons of European ancestry (Table 1). The missense mutations are predicted to have a deleterious effect on protein function (Table S3 in the Supplementary Appendix). The mutant amino acids are located in the second immunoglobulin-like domain or the catalytic domain of the enzyme (Fig. 1D). Protein modeling suggested that these mutations would be likely to result in protein misfolding (Fig. S2 in the Supplementary Appendix).[17,18] The splice-site mutation c.832-2A→G is expected by several prediction tools[19-22] to abrogate the acceptor splice site of intron 7 and to effect skipping

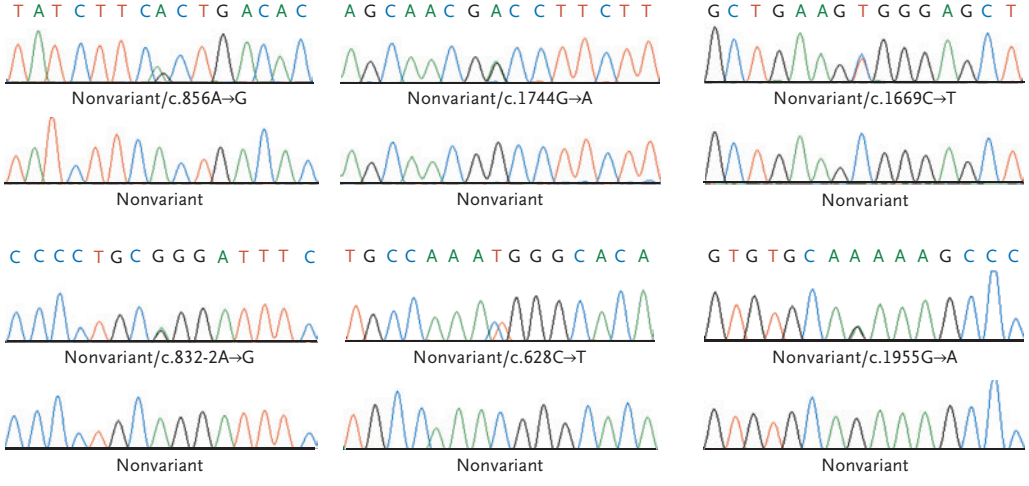
A CCCA in Patient 29



B Histopathological Results in Patient 11



C Direct Sequencing Results of Heterozygous Mutations in *PADI3* vs. Nonvariant *PADI3*



D Amino Acid Substitutions Predicted by Missense Variants in *PADI3*

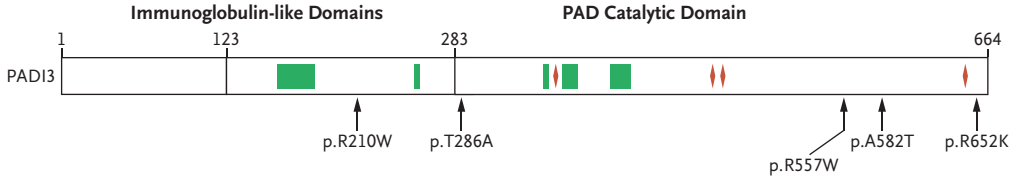


Figure 1 (facing page). Clinical Features and Mutation Analysis in Central Centrifugal Cicatricial Alopecia (CCCA).

Patient 29 presented with scarring alopecia involving the central part of the scalp, which was mostly prominent over the vertex area; tufted hairs were visible (Panel A) (Table S2 in the Supplementary Appendix). Histopathological examination of a transverse section (isthmus level) of a scalp-skin biopsy sample obtained from Patient 11 (referred to as Patient II-1 in Family 1) showed markedly decreased follicular density (Panel B; arrows indicate hair follicles), perifollicular concentric fibrosis, and eccentric thinning (epithelial atrophy) of the follicular epithelium (Fig. S1 in the Supplementary Appendix). Direct sequencing of *PADI3* revealed six heterozygous mutations: c.856A→G (p.Thr286Ala [p.T286A]), c.1744G→A (p.Ala582Thr [p.A582T]), c.1669C→T (p.Arg557Trp [p.R557W]), c.832-2A→G, c.628C→T (p.Arg210Trp [p.R210W]), and c.1955G→A (p.Arg652Lys [p.R652K]). For comparison, the corresponding nonvariant sequences are shown below each mutated sequence (Panel C). The location of the amino acids predicted by the five missense variants is shown along a schematic representation of the PADI3 protein, its domains, the positions of calcium-binding sites (green rectangles), and the major amino acids involved in the catalytic sites (red diamonds) (Panel D). PAD denotes peptidyl–arginine deiminase domain.

of exon 8, which in turn is expected to lead to a frame shift.

CONSEQUENCES OF CCCA-ASSOCIATED MUTATIONS IN *PADI3*

PADI3[17] is a member of the peptidyl arginine deiminase family of enzymes, which are responsible for catalyzing the post-translational deimination of proteins by converting positively charged L-arginine residues into citrullines in the presence of calcium ions.[23] They have distinct substrate specificities and tissue-specific expression patterns.[23,24] PADI3 is detected mainly in the epidermis and hair follicles.[25,26] In the skin, it is responsible for mediating the modification of proteins critical for normal hair-shaft formation and shaping, such as trichohyalin, and may also play a role in interfollicular epidermal differentiation.[23]

Although PADI3 has been associated with abnormal hair formation in patients who have the uncombable hair syndrome (Online Mendelian Inheritance in Man number, 191480),[9] it has been unclear whether it has a role in the pathogenesis of CCCA. In an attempt to obtain further in vivo evidence of the relevance of CCCA-associated mutations to the disease manifestations, we used deep sequencing of RNA extracted from biopsy samples of scalp skin obtained from three patients with CCCA who had mutations in *PADI3* and from four healthy controls who were matched for ancestry population, age, and sex.

The expression of numerous genes differed between scalp-skin samples obtained from patients with CCCA and control samples (Fig. S3A and Table S4 in the Supplementary Appendix). Expression of *PADI3* was markedly lower in the skin of patients with CCCA than in the skin of controls (Table S4 in the Supplementary Appendix), as was the expression of genes encoding several peptidyl arginine deiminase substrates (including *TCHH*[9] and *S100A3*[27]), those known to be related to hair loss (including *LIPH*,[28] *DSG4*,[29] *HR*,[30] and *CDSN*[31]), and those encoding hair keratins and keratin-associated proteins (which contribute to the normal structure of hair fibers[32]). Ingenuity pathway analysis revealed that the expression of many genes encoding molecules that play a central role in hair-follicle development was reduced overall in the skin of patients with CCCA. Relevant RNA-sequencing data were validated with the use of quantitative reverse-transcriptase polymerase chain reaction. Details are provided in Figures S3 through S5 in the Supplementary Appendix.

To further investigate the consequences of the CCCA-associated missense mutations in *PADI3*, HaCaT (a human keratinocyte cell line) cells were transiently transfected with constructs encoding nonvariant and mutated *PADI3*. Immunoblotting of cell extracts showed expression of all three mutant PADI3 constructs that was slightly lower than that of the nonvariant construct (Fig. 2A). Accordingly, PADI3 expression was reduced in a scalp-skin sample obtained from a patient with CCCA (Fig. 2B). We then examined the effect of *PADI3* mutations on the subcellular location of the enzyme. Immunofluorescence analyses showed a homogeneous cytoplasmic distribution of PADI3 in cells transfected with nonmutated *PADI3*, as previously shown,[9] in contrast with cells transfected (one at a time) with the three mutated *PADI3* variants. In these cells, we observed abnormal

Table 1. Variants Identified in *PADI3* in Patients with Central Centrifugal Cicatricial Alopecia.

DNA Sequence Variant	Amino Acid Sequence Change	Study Patients with Mutation*	Minor Allele Frequency†		ClinVar Accession No.
			African Population	European Population	
		patient number			
c.856A→G	p.Thr286Ala	1, 3, 9, 16, 47, 50	0.03647	0.00010	SCV000863555
c.1744G→A	p.Ala582Thr	3, 17, 58	0.02267	0.00004	SCV000863556
c.1669C→T	p.Arg557Trp	11, 31, 52	0.00753	0.00007	SCV000863557
c.832-2A→G	Splicing	11, 52	0.00000	0.00000	SCV000863558
c.1955G→A	p.Arg652Lys	49	0.00011	0.00000	SCV000863559
c.628C→T	p.Arg210Trp	54, 55	0.00164	0.00005	SCV000863560

* Details regarding the patients in the study are provided in Table S2 in the Supplementary Appendix.
† Data regarding the minor allele frequencies are from the Genome Aggregation Database (http://gnomad.broadinstitute.org/).

intracellular localization of the protein with formation of aggregates in the cytoplasm (Fig. 2C).

We then assayed enzymatic activity associated with the three mutant constructs, as compared with nonvariant *PADI3*. A construct with a mutation that had been previously associated with the uncombable hair syndrome[33] served as a positive control. We observed a significant decrease in enzymatic activity on transfection of the four constructs into HaCaT cells, as compared with the HaCaT cells transfected with the construct containing nonmutated *PADI3* (Fig. 2D).

FREQUENCY OF *PADI3* MUTATIONS IN CCCA

We then sequenced *PADI3* in a replication set, which included 42 patients (Table S2 in the Supplementary Appendix); we observed a *PADI3* variant in 9 of them. Altogether, we identified a total of six different mutations in *PADI3* (Fig. 1C and Table 1), which were present in 14 of the 58 patients (24%) with CCCA who participated in this study. The two familial cases were identified; these patients were members of families with cosegregation of the mutations and affected status (Fig. S1 in the Supplementary Appendix).

In a post hoc analysis, the *PADI3* mutation frequency among 58 women of African ancestry who had CCCA (116 alleles) was found to differ significantly from that calculated for a control cohort of women of African ancestry (from the gnomAD V2.1 control set) according to the chi-square test (P=0.002) and Fisher's exact test (P=0.006). The difference remained significant after adjustment for relatedness of persons according to the chi-square test (P=0.03) and Fisher's exact test (P=0.04). We did not control for population stratification. However, the mutation frequency was similar across various African subpopulations (Table S5 in the Supplementary Appendix).

DISCUSSION

Loss-of-function mutations in *PADI3* were associated with CCCA in women of African ancestry. PADI3 mediates deimination of hair structural proteins such as S100A3 in the cuticle[27] and trichohyalin in the medulla and the Henle layer of the inner root sheath.[34] Previous observations have pointed to the importance of PADI3 for proper hair-shaft formation: *Padi3*-knockout mice have whisker and hair anomalies,[9] biallelic mutations in *PADI3* in humans are associated with autosomal recessive uncombable hair syndrome,[9] and *PADI3* variants are major determinants of hair-shaft shape.[35] Taken together, these data predict that decreased expression, diminished activity, or misfolding of PADI3 is likely to exert a deleterious effect on hair-shaft formation and hair-follicle development, which may underlie the disease phenotype seen in CCCA.

The finding that 14 of 58 patients (24%) in our

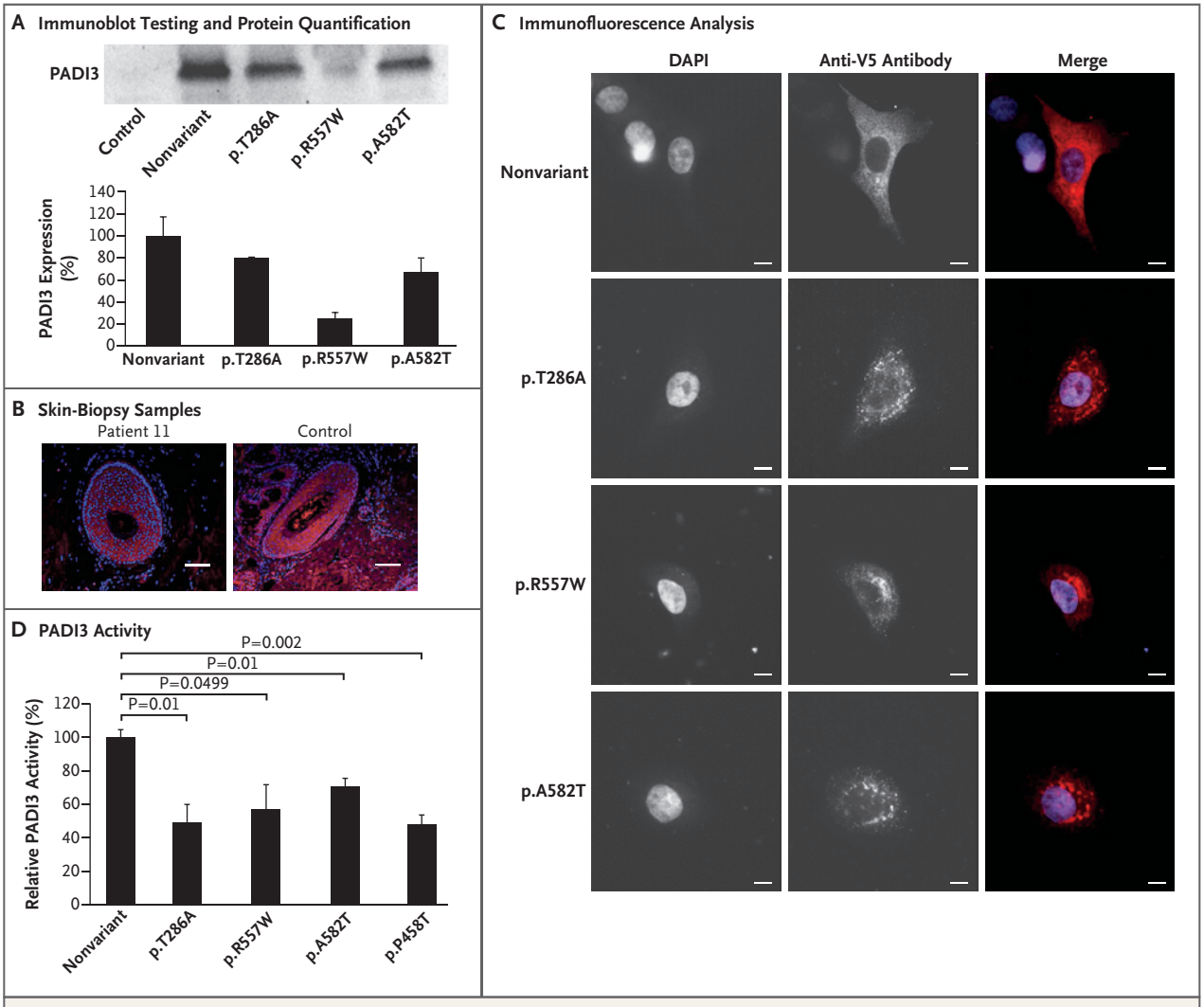


Figure 2. Consequences of CCCA-Associated Variants in *PADI3*.

HaCaT cells (a human keratinocyte cell line) were transfected with plasmids expressing either nonvariant *PADI3* V5-tagged complementary DNA (cDNA) or *PADI3* V5-tagged cDNA with p.Thr286Ala (p.T286A), p.Arg557Trp (p.R557W), and p.Ala582Thr (p.A582T) mutations. Immunoblotting analysis was used to ascertain the expression of the four *PADI3* constructs in transiently transfected HaCaT cells 48 hours after transfection. Immunoblotting of total protein extracts was performed with an anti-V5 antibody (Panel A, upper panel), and the protein intensity was quantified (lower panel). Results are expressed as a percentage of PADI3 expression relative to the expression of the nonvariant PADI3. Results represent the mean of two independent experiments. T bars indicate the standard error. Skin-biopsy samples obtained from Patient 11 (from Family 1) and from a healthy person (control) were stained with an anti-PADI3–specific antibody (scale bar, 50 μm) (Panel B). Immunofluorescence analysis was performed in HaCaT cells that were transiently transfected with the four *PADI3* constructs (Panel C). Whereas nonvariant PADI3 shows homogeneous cytosolic localization, the three mutant proteins are observed in the form of large aggregates (scale bar, 10 μm). DAPI denotes 4′,6-diamidino-2-phenylindole. HaCaT cells were transfected with plasmids expressing either nonvariant *PADI3* cDNA or *PADI3* cDNA with p.T286A, p.R557W, and p.A582T mutations (Panel D). The mutation p.Pro458Thr (p.P458T), which has previously been shown to cause the uncombable hair syndrome, was used as a positive control. PADI3 activity was measured with the use of an antibody-based assay for PAD activity according to the manufacturer's instructions (see the Supplementary Methods section in the Supplementary Appendix). Results are expressed as a percentage of PADI3 activity relative to the activity of the nonvariant PADI3. Results represent the mean of three independent experiments, which were assessed with the use of two-sided Student's t-tests. T bars indicate the standard error.

study had mutations in the *PADI3* coding sequence suggests that the disease is genetically heterogeneous, which in turn may underlie the varying clinical manifestations of CCCA.[1,2] As mentioned, biallelic mutations in *PADI3* have been shown to cause the uncombable hair syndrome,[9] and so this syndrome and CCCA are allelic disorders. It is relevant to note that the *PADI3* mutations associated with CCCA differ from those responsible for the uncombable hair syndrome. In contrast to the uncombable hair syndrome, which is characterized by an early age of onset, autosomal recessive inheritance, and substantial clinical manifestations,[36] CCCA is characterized by late onset, dominant inheritance, and varying severity.[1,3] These differences may be due to the fact that although CCCA-associated mutations result in decreased expression and mislocalization of PADI3, they mostly occur only on one allele of the gene.

CCCA disproportionately affects persons of African ancestry. Hair in persons of African ancestry has fewer elastic fibers, which anchor the hair follicles,[37] and differs from that of persons of other ancestries in that it is tightly coiled and has a flattened cross-sectional appearance. (Persons of European and Asian ancestry have hair with oval and circular cross-sectional appearance, respectively.[38]) Although the keratin fiber structure seems to be similar in hair of persons of African, European, and Asian ancestries, the structure in persons of African ancestry is more fragile.[39] These differences combined with hair-grooming habits[40] and hereditary factors,[8] such as damaging genetic variants in *PADI3*, may account for the greater percentage of women of African ancestry with CCCA (as compared with women of other ancestries), although it is not possible, with the data to hand, to determine whether this is true. The different properties of hair among persons of African ancestry and those of European ancestry may explain, in part, the different clinical consequences of *PADI3* mutations in CCCA and in the uncombable hair syndrome. Alternatively, the distinct variants in *PADI3* in each of the disorders may account for the difference in clinical outcomes.

The reason for the persistence and accumulation of prevalent and damaging genetic variants in *PADI3* in persons of African ancestry remains to be elucidated. Perhaps these variants confer a survival or reproductive advantage as has been shown previously for variants in other genes.[41,42]

In conclusion, we found that CCCA was associated with deleterious variants in *PADI3*, which encodes a critical mediator of hair formation.

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Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

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