

Loss-of-Function Mutations in the *Filaggrin* Gene and Alopecia Areata: Strong Risk Factor for a Severe Course of Disease in Patients Comorbid for Atopic Disease

Regina C. Betz¹, Jana Pffor¹, Antonia Flaquer², Silke Redler¹, Sandra Hanneken³, Sibylle Eigelshoven³, Anne-Katrin Kortüm³, Thomas Tüting⁴, Julien Lambert⁵, Jozef De Weert⁶, Axel M. Hillmer⁷, Christine Schmael⁸, Thomas F. Wienker², Roland Kruse³, Gerhard Lutz⁹, Bettina Blaumeiser¹⁰ and Markus M. Nöthen⁷

Alopecia areata (AA) is a common dermatological disease, which affects nearly 2% of the general population. Association of AA with atopic disease has been repeatedly reported. Loss-of-function mutations in the *filaggrin* gene (*FLG*) may be considered as promising candidates in AA, as they have been observed to be a strong risk factor in atopic dermatitis. The *FLG* mutations R501X and 2282del4 were genotyped in a large sample of AA patients ($n = 449$) and controls ($n = 473$). Although no significant association was observed in the patient sample overall, *FLG* mutations were significantly associated with the presence of atopic dermatitis among AA patients. Furthermore, the presence of *FLG* mutations had a strong impact on the clinical course of AA in comorbid patients. For example, 19 of the 22 mutation carriers among AA patients with atopic dermatitis showed a severe form of the disease ($P = 0.003$; odds ratio (OR) = 5.47 (95% confidence interval (CI): 1.59–18.76)). In conclusion, our data suggest that when AA occurs in conjunction with *FLG*-associated atopic disorder, the clinical presentation of AA may be more severe.

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INTRODUCTION

Alopecia areata (AA) is a common skin disease presenting with patchy hair loss, which affects approximately 1–2% of the general population (Safavi *et al.*, 1995). Its etiopathogenesis is incompletely understood, but it is thought to be a tissue-specific autoimmune disease directed against the hair follicle.

Family and twin studies suggest strongly that genetic factors, which probably act in a polygenic fashion, play an important role in the development of AA (Jackow *et al.*, 1998;

Blaumeiser *et al.*, 2006). Various genes related to immune response have been postulated to be associated with AA (Galbraith and Pandey, 1995; Tazi-Ahmini *et al.*, 2000, 2002a, b; Barahmani *et al.*, 2002), but only the involvement of the major histocompatibility complex has been confirmed through independent replication (Duvic *et al.*, 1995; Colombe *et al.*, 1999; de Andrade *et al.*, 1999; Entz *et al.*, 2006).

An association of AA with atopic disease has been reported in several studies (Ikeda, 1965; Young *et al.*, 1978; Tan *et al.*, 2002; Goh *et al.*, 2006). Mutations in the *filaggrin* gene (*FLG*) have been observed to be a strong risk factor for atopic dermatitis (Palmer *et al.*, 2006; Ruether *et al.*, 2006; Weidinger *et al.*, 2006; Stemmler *et al.*, 2007; Barker *et al.*, 2007; Morar *et al.*, 2007; Weidinger *et al.*, 2007), and we therefore hypothesized that *FLG* mutations may also play a role in AA, particularly in those patients with comorbid atopic disease. As comorbidity with atopic disease has been associated previously with a severe form of AA (Ikeda, 1965; Goh *et al.*, 2006), we also wished to test whether this effect, if present in our sample, is influenced by the presence of *FLG* mutations.

RESULTS

The two previously reported loss-of-function mutations of the *FLG* gene, R501X and 2282del4, were genotyped in our cases and controls. The genotype distributions of the two mutations

¹Institute of Human Genetics, University of Bonn, Bonn, Germany; ²Institute for Medical Biometry, Informatics and Epidemiology, University of Bonn, Bonn, Germany; ³Department of Dermatology, University of Düsseldorf, Düsseldorf, Germany; ⁴Department of Dermatology, University of Bonn, Bonn, Germany; ⁵Department of Dermatology, University of Antwerp, Antwerp, Belgium; ⁶Department of Dermatology, University of Gent, Gent, Belgium; ⁷Department of Genomics, Life and Brain Center, University of Bonn, Bonn, Germany; ⁸Division of Genetic Epidemiology in Psychiatry, Central Institute of Mental Health, Mannheim, Germany; ⁹Hair and Nail, Bonn, Germany and ¹⁰Department of Medical Genetics, University of Antwerp, Antwerp, Belgium

Correspondence: Dr Regina C. Betz, Institute of Human Genetics, University of Bonn, Wilhelmstrasse 31, Bonn D-53111, Germany.
E-mail: regina.betz@uni-bonn.de

Abbreviations: AA, alopecia areata; AT, alopecia totalis; AT/AU, alopecia totalis/universalis; AU, alopecia universalis; FH+, family history positive; FH–, family history negative; *FLG*, *filaggrin* gene

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observed in patients and controls are shown in Table 1. Distributions of genotypes were consistent with Hardy–Weinberg equilibrium for 2282del4 in both groups and for R501X in controls. The distribution of genotypes for R501X significantly departed from Hardy–Weinberg proportions in patients ($P=6.4 \times 10^{-7}$).

In control chromosomes, mutation frequencies were 1.5% for R501X and 2.4% for 2282del4. This is similar to frequencies reported in other European populations (Palmer *et al.*, 2006). Two patients were homozygous for R501X and two patients were compound heterozygotes for R501X and 2282del4. No significant differences in genotype or allele frequencies between patients and controls were observed for either of the two mutations (Table 1). It is interesting to note, however, that all homozygotes and compound heterozygotes

were observed among patients. Given the rarity of these genotypes, no real conclusions can be drawn concerning a possible effect of these genotypes on the risk to develop AA. As the two mutations are considered to be functionally equivalent, and since the observed effects of the two mutations in our sample were similar, we combined the two mutations for the purposes of the detailed analyses (Table 2). Only patients for whom both mutations had been characterized were included in the analyses.

Thirty-four percent of our patients had a history of atopic dermatitis (145/430). Among them were 37 patients with a history of atopic dermatitis and asthma, and 27 with a history of atopic dermatitis, asthma and allergic rhinitis. We found highly significant differences in the frequency of *FLG* mutations in patients with atopic dermatitis ($P=0.005$),

Table 1. Frequency of *FLG* mutations R501X and 2282del4 in alopecia areata patients and controls

		Genotype distribution (%)			P-value ¹	Allele frequency		P-value ²
		AA	Aa	aa		A	a	
R501X	Controls (n=469)	455 (97.0)	14 (3.0)	0	0.730	0.985	0.015	0.713
	Patients (n=444)	430 (96.8)	12 (2.7)	2 (0.5)		0.982	0.018	
2282del4	Controls (n=458)	436 (95.2)	22 (4.8)	0	0.123	0.976	0.024	0.129
	Patients (n=435)	403 (92.6)	32 (7.4)	0		0.963	0.037	

¹P-value from the Cochran–Armitage trend test. P-values were not corrected for multiple testing.

²P-value from the Fischer’s exact test. P-values were not corrected for multiple testing.

Table 2. Frequency of combined *FLG* null mutations in alopecia areata patients, their subgroups, and controls

	Genotype distribution (%)			P-value ¹	Allele frequency		P-value ²	Odds ratio (95% CI)
	AA	Aa	aa		A	a		
Controls (n=449)	413 (92.0)	36 (8.0)	0		0.960	0.040		
Patients (n=430)	386 (89.8)	40 (9.3)	4 (0.9)	0.156	0.944	0.056	0.146	1.42 (0.91–2.20)
Atopic dermatitis (n=145)	123 (84.8)	20 (13.8)	2 (1.4)	0.006	0.917	0.083	0.005	2.16 (1.27–3.69)
Atopic dermatitis+asthma (n=37)	26 (70.3)	10 (27.0)	1 (2.70)	0.001	0.838	0.162	0.0001	4.63 (2.30–9.35)
Atopic dermatitis+asthma+allergic rhinitis (n=27)	19 (70.4)	7 (25.9)	1 (3.7)	0.001	0.833	0.167	0.001	4.79 (2.17–10.55)
Mild AA ³ (n=192)	178 (92.7)	14 (7.3)	0	0.873	0.964	0.037	0.875	0.91 (0.48–1.70)
Severe AA ⁴ (n=238)	208 (87.4)	26 (10.9)	4 (1.7)	0.017	0.929	0.071	0.014	1.84 (1.14–2.98)
FH+ ⁵ (n=120)	109 (90.8)	10 (8.3)	1 (0.8)	0.588	0.950	0.050	0.587	1.26 (0.65–2.46)
FH– ⁶ (n=306)	274 (89.5)	29 (9.5)	3 (1.0)	0.145	0.943	0.057	0.138	1.45 (0.90–2.34)
Onset age ≤20 (n=194)	177 (91.2)	16 (8.3)	1 (0.5)	0.649	0.954	0.046	0.650	1.16 (0.65–2.08)
Onset age >20 (n=236)	209 (88.6)	24 (10.2)	3 (1.3)	0.688	0.936	0.064	0.063	1.63 (0.99–2.67)

¹P-value from the Cochran–Armitage trend test. P-values were not corrected for multiple testing.

²P-value from the Fischer’s exact test. P-values were not corrected for multiple testing.

³Patients with patchy alopecia areata (one or more circumscribed patches of hair loss).

⁴Patients with alopecia totalis (100% loss of scalp hair without loss of body hair), alopecia totalis/universalis (100% scalp hair loss with variable loss of body hair), and alopecia universalis (100% of both scalp and body hair).

⁵Defined as history of at least one first- or second-degree relative with alopecia areata.

⁶Defined as no history of first- or second-degree relative with alopecia areata.

atopic dermatitis + asthma ($P=0.0001$), and atopic dermatitis + asthma + allergic rhinitis ($P=0.001$). The associated odds ratios (ORs) are given in Table 2. Subdividing patients according to family history of AA (FH+, FH-) and age at onset revealed no significant associations in the respective subgroups (Table 2).

In a subsequent step, we tested the effect of comorbidity and *FLG* mutations on the severity of AA (Table 3, see Materials & Methods for detailed description of phenotype classification). While the frequency of atopic dermatitis was similar among mildly and severely affected individuals ($P=0.474$), the combination of atopic dermatitis + asthma ($P=0.009$) and atopic dermatitis + asthma + allergic rhinitis ($P=0.001$) was significantly associated with a severe course of disease. To test whether the presence of *FLG* mutations identifies a subgroup of atopic disease more likely to have severe AA, we analyzed mutation carriers and non-carriers separately. The presence of *FLG* mutations among comorbid patients was strongly associated with a severe course of the disease (Table 3). The odds ratio among mutation carriers raised from 5.47 (1.59–18.76, $P=0.003$) in AA patients with atopic dermatitis to 8.38 (1.06–66.03, $P=0.027$) in patients with atopic dermatitis + asthma and 14.20 (0.81–247.6, $P=0.01$) in patients with atopic dermatitis + asthma + allergic rhinitis. In non-carriers, only a small effect was seen in the subgroup of patients with atopic dermatitis + asthma + allergic rhinitis (OR=3.16 (95% CI: 1.03–9.69), $P=0.036$).

DISCUSSION

The results of our study clearly demonstrate that *FLG* mutations are observed at an increased frequency among AA patients with a history of atopic dermatitis. This finding had been anticipated since previous work has suggested that atopic dermatitis is the core phenotype associated with *FLG* mutations (Palmer *et al.*, 2006). Thirty-four percent of the AA patients in our sample were comorbid for atopic dermatitis. This high rate of comorbidity supports previous observations of an association between AA and atopic disorders (Ikeda,

1965; Young *et al.*, 1978; Tan *et al.*, 2002; Goh *et al.*, 2006). However, the comorbidity rate observed in our sample may not be an accurate reflection of the true comorbidity between the two disorders. First, history of an atopic disorder was assessed in our sample by taking clinical history, without confirmation through laboratory tests or review of medical records. We believe, however, that (1) the rate of diagnostic misspecification was small, as the clinical interviews were performed by an experienced medical doctor who asked additional questions if information from patients was unclear and (2) the frequency of *FLG* mutations in our AA patients with atopic dermatitis (combined carrier frequency of 15.2%) was similar to the frequency observed in a large sample of well-characterized atopic dermatitis patients of mainly German origin (combined carrier frequency of 16.7%) (Marenholz *et al.*, 2006). Second, the majority of our patients were recruited from a clinical setting, which may have resulted in a higher rate of comorbidity than is the case in population-based samples.

Comorbidity with atopic disease has been associated previously with a severe form of AA (Ikeda, 1965; Goh *et al.*, 2006), and we can confirm this for the groups of patients with atopic dermatitis + asthma and atopic dermatitis + asthma + allergic rhinitis. Our molecular data suggest that this effect is driven almost exclusively by the presence of *FLG* mutations. In the absence of *FLG* mutations, comorbidity with atopic disorder has no or very little effect on the severity of AA. Filaggrin plays an important role as a protein in the formation of the epidermal barrier, through binding to and aggregating with the keratin cytoskeleton (Irvine and McLean, 2006). Our findings therefore suggest that an epidermal barrier defect contributes to a severe course of AA.

MATERIALS AND METHODS

Subjects

Four hundred and forty-nine AA patients (298 women and 151 men) aged 5–82 years (mean age: 52.5) were recruited from the outpatient hair clinics of four Departments of Dermatology, that is the University Hospitals at Antwerp and Gent ($n=260$), Düsseldorf

Table 3. Association of *filaggrin* mutations with severity of AA in comorbid patients

	Patchy AA (n=192)		AT/AU (n=238)		P-value ¹	Odds ratio (95% CI)
	n	%	n	%		
Atopic dermatitis	61	31.8	84	35.3	0.474	1.17 (0.78–1.75)
Filaggrin null	3	1.6	19	8.0	0.003	5.47 (1.59–18.76)
Filaggrin wt	58	30.2	65	27.3	0.521	0.87 (0.57–1.32)
Atopic dermatitis+asthma	9	4.7	28	11.8	0.009	2.71 (1.25–5.89)
Filaggrin null	1	0.5	10	4.2	0.027	8.38 (1.06–66.03)
Filaggrin wt	8	4.2	18	7.6	0.159	1.88 (0.80–4.43)
Atopic dermatitis +asthma+allergic rhinitis	4	2.1	23	9.7	0.001	5.03 (1.71–14.80)
Filaggrin null	0	0.0	8	3.4	0.01	14.20 ² (0.18–247.6)
Filaggrin wt	4	2.1	15	6.3	0.036	3.16 (1.03–9.69)

AA, alopecia areata; AT/AU, alopecia totalis and/or universalis, CI, confidence interval.

¹P-value from the Fischer's exact test. P-values were not corrected for multiple testing.

²Because one of the cells contains a zero, the OR has been computed adding 0.5 to each cell.

($n=107$), and Bonn ($n=17$) and from a private dermatology practice in Wesseling ($n=65$). Clinical data were obtained from all patients, including age at onset and family history. AA type was determined according to the AA investigational assessment guidelines (Olsen *et al.*, 2004) and patients were categorized as having either patchy alopecia, alopecia totalis (AT), alopecia totalis/universalis (AT/AU), or alopecia universalis (AU). Patchy alopecia is defined as one or more circumscribed patches of hair loss, AT is defined as 100% loss of scalp hair without loss of body hair, AT/AU is defined as 100% scalp hair loss with variable loss of body hair, and AU is defined as 100% loss of both scalp and body hair. Assessment of severity was based on lifetime perspective and according to the most severe episode ever experienced. The AT, AT/AU, and AU patients included in our study were classified as having severe AA, whereas the patchy AA patients were classified as having mild AA. Two hundred patients presented with patchy AA, 34 with AT, 26 with AT/AU, and 187 with AU. 126/449 patients reported a family history of AA (28.1%), defined as having at least one first- or second-degree relative with AA (Blaumeiser *et al.*, 2006).

A personal history of atopic disease was determined by asking patients whether they had ever suffered from atopic dermatitis, allergic rhinitis, and asthma.

The control group was comprised of 473 healthy unrelated sex and age-matched anonymous blood donors. Patients and controls were all of Central European origin.

Ethical approval for the study was obtained from the Ethics Committees of the University Hospitals Bonn, Düsseldorf, and Antwerp. Written informed consent was obtained before taking blood from AA patients and controls. The study was conducted in concordance with the Declaration of Helsinki Principles.

Laboratory methods

DNA was extracted from peripheral blood leukocytes according to standard methods. PCR fragments were amplified as described previously (Smith *et al.*, 2006). The mutation R501X creates a new restriction site *Nla*III (= *Hin*III, Fermentas) that was used before resolving the digest on 13% polyacrylamide gels (49:1). The digest of the 2282del4 mutation was performed with *Dra*III (= *Adel*, Fermentas) and resolved on 3% agarose gels. The genotype of the two individuals homozygous for R501X was confirmed by sequencing using independent pairs of PCR primers.

Statistical analysis

Genotype frequencies of the cases and controls were tested for deviation from Hardy–Weinberg equilibrium using the χ^2 -test (1 df). Genotypic distributions between cases and control subjects were compared using the Cochran–Armitage trend test (Cochran, 1954; Armitage, 1955). The trend test is a method of directing χ^2 -test toward narrow alternatives. The test is sensitive to the linearity between response variable and experimental variables and detects trends that would remain undetected by cruder methods (Sasieni, 1997). In addition, the trend test is still valid when genotype frequencies do not comply with Hardy–Weinberg proportions. Allele frequencies were compared with the Fisher's exact test (Fisher, 1922). All these tests are included in the statistical package SAS version 9.1 (SAS Institute, Cary, NC).

Some of our subgroup analyses were independent from testing of the primary hypotheses. We did not correct for multiple testing, as we considered these to be exploratory analyses. Independent replication of these results is therefore needed.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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