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Polymorphisms of the filaggrin gene are associated with atopic dermatitis in the Caucasian population of Central Russia

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ABSTRACT

Association of the filaggrin (*FLG*) gene with atopic dermatitis (AD) in Caucasians from Central Russia was studied in the sample of 700 patients and 612 controls. In total ten SNPs of the gene (rs61816761, rs12130219, rs77199844, rs558269137, rs4363385, rs12144049, rs471144, rs6661961, rs10888499, rs3126085), their haplotypes and interlocus interactions were analyzed using logistic regression. The functional effects of the AD risk candidate loci and their proxies (136 SNPs) were evaluated by *in silico* analysis. All analyzed SNPs were associated with AD: two SNPs (rs3126085 and rs12144049) manifested the independent association, nine SNPs were associated within 30 haplotypes, and seven SNPs showed interlocus interaction effects within ten most significant epistatic models. Alleles A rs3126085 and C rs12144049 were associated with a higher risk of AD according to the allelic (ORs being 1.75, $p_{perm} = 0.002$ and 1.45, $p_{perm} = 0.011$ respectively), additive (ORs being 1.69, $p_{perm} = 0.004$ and 1.47, $p_{perm} = 0.011$ respectively) and dominant (ORs being 1.79, $p_{perm} = 0.004$ and 1.63, $p_{perm} = 0.005$ respectively) genetic models. Three haplotypes, GT[rs3126085-rs12144049] (OR = 0.60), GGT[rs61816761-rs3126085-rs12144049] (OR = 0.59), and AWGGT[rs12130219-rs558269137-rs61816761-rs3126085-rs12144049] (OR = 0.63) demonstrated the protective effect ($p_{perm} = 0.001$). The *in silico* analysis suggested that the AD risk variants and their proxies apparently produce various effects on 38 genes in various tissue/organs (including 20 genes in the skin). The biological process enrichment analyses suggest that the target AD candidate genes influence the formation of the cornified envelope, keratinization and cornification, and more than twenty other pathways related to skin development, programmed cell death, and regulation of water loss via skin.

1. Introduction

Atopic dermatitis (AD) or eczema (OMIM 603165) is an acute or chronic (recurrent) non-contagious skin disease caused by serous inflammation of the predominantly papillary dermis and focal spongiosis of the prickly epidermis, manifested by a polymorphically itchy rash (Bieber, 2008). Lesions typically manifest age-related morphology and distribution (Eichenfield et al., 2014). The AD prevalence was reported to be 7.2–10.2% in adults (Silverberg, 2017). AD substantially affects the psychosocial well-being and patient's quality of life (Dalgard et al., 2015). The risks of insomnia, anxiety, and depression in individuals suffering from AD are respectively 79%, 44%, and 41% higher as compared with the general population (Chidwick et al., 2020). In adults with atopic dermatitis, the prevalence of suicidal ideation exceeds 20% (Dieris-Hirche et al., 2017). Treatment of AD incurs significant costs for patients, their families, and society (Adamson, 2017; Drucker et al., 2017).

The pathophysiology of AD is still unclear but existing evidence suggests skin barrier dysfunction and immune dysregulation as the most apparent causes of it (Kim et al., 2019; Brunner et al., 2018). Indeed, the epidermis is a key physical and functional barrier, and skin barrier defects are the most common pathologic formations in the AD skin (Egawa and Kabashima, 2016; Kim and Leung, 2018). Several proteins, such as filaggrin (FLG), keratins, transglutaminases, and intercellular proteins

Abbreviations: AD, atopic dermatitis; FLG, filaggrin; GWAS, genome-wide association studies; MB-MDR, Model-Based Multifactor Dimensionality Reduction; FDR, false discovery rate; LD, linkage disequilibrium.

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were implicated in the epidermal function. Therefore, defects in these proteins may facilitate penetration of allergens and microbes into the skin (Egawa and Kabashima, 2016).

Atopic dermatitis has a significant genetic component with up to 90% heritability as estimated in Europeans (Bataille et al., 2012). The null mutations in the *FLG* gene (e.g., R501X, 2282del4) resulting in the epidermal barrier deficiency were recognized among the strongest known risk factors (Palmer et al., 2006; Irvine et al., 2011). Genome-wide association studies (GWAS) have identified several additional susceptibility loci of the *FLG* gene contributing to AD (Sun et al., 2011; Weidinger et al., 2013; Paternoster et al., 2015; Marenholz et al., 2015; Baurecht et al., 2015; Schaarschmidt et al., 2015). However, among the AD candidate polymorphisms of the *FLG* gene, only one, rs12144049, was replicated in two GWAS (Baurecht et al., 2015; Schaarschmidt et al., 2015). This prompts for more replication studies of the *FLG* gene variants in various ethnic populations.

The purpose of this study was to replicate the association of ten variants in the *FLG* gene (including GWAS-significant SNPs and null mutations) with AD in a Caucasian population from the Central Region of Russia.

2. Subjects and methods

2.1. Study subjects

The study protocol was approved by the Ethical Review Committee of Belgorod State University. All participants signed an informed consent prior to enrolment in the study. All research was performed in accordance with relevant per the principles of the Helsinki Declaration. In total, 1312 participants of Russian origin, born and living in the Central region of Russia (Litovkina et al., 2014; Reshetnikov et al., 2015) including 700 CE patients and 612 controls, were recruited at Belgorod and Kursk regions dermatovenerologic dispensaries during the 2010–2018 period. The UK Diagnostic Criteria was applied by qualified dermatologists to diagnose AD (Williams et al., 1994). AD severity was assessed using the Eczema Area and Severity Index (EASI) (Hanifin et al., 2001). The control group consisted of healthy individuals without symptoms of AD, other skin and atopic diseases (asthma, hay fever, allergic conjunctivitis, sensitization to allergens (air pollutants, food, medication, domestic animals, indoor allergens, etc.)), a family history of atopic diseases (Belyaeva, 2020). All participants (cases and controls) had no oncological, severe autoimmune, and chronic vital organ diseases (lung, heart, or renal failure) (Ponomarenko et al., 2020a).

2.2. Isolation DNA, selection SNPs, and genotyping procedure

A sample of whole blood (4–5 ml) was collected by venipuncture from each participant in EDTA-containing Vacutainer® tubes (Moskalenko et al., 2019). DNA was extracted from the buffy coat to apply the generally accepted phenol/chloroform procedure (as described earlier (Ponomarenko et al., 2020b)).

Ten SNPs of the *FLG* gene (rs12130219, rs558269137, rs61816761, rs3126085, rs12144049, rs6661961, rs471144, rs10888499, rs77199844, rs4363385) were selected for the associations analysis. To select SNPs, we used the following criteria (Reshetnikov et al., 2017; Ponomarenko et al., 2019): previously reported associations with AD (eczema), other skin (psoriasis, ichthyosis vulgaris) and some allergic disorders (asthma, hay fever) and regulatory potential.

All ten selected SNPs had impact regulatory effects (the functionality of the SNPs was assessed *in silico* by the HaploReg database (Ward and Kellis, 2016)) (Table S15) and were associated with AD (eczema) in previously published candidate gene association studies including nine GWAS-significant SNPs (Table S16). Also, five SNPs were previously associated with some skin and others allergic disorders (psoriasis, asthma, hay fever, etc.) (Table S16).

SNPs *FLG* gene genotyping was conducted using the MALDI-TOF mass spectrometry iPLEX platform (Agena Bioscience Inc, San Diego, CA). Blind replicates were genotyped for quality control (Golovchenko et al., 2020). Regenotyping of 5% randomly selected studied samples showed 100% reproducibility of the original results.

2.3. Statistical analysis

The observed genotype and allele distribution by the chi-square test were assessed for correspondence to the Hardy-Weinberg equilibrium. Odds ratio (OR) and 95% confidence intervals (95% CI) (Minyaylo et al., 2021) were calculated to estimate the association between the *FLG* gene polymorphisms and AD risk used logistic regression (allelic, recessive, additive, and dominant genetic models were tested) and adjusted for covariates such as sex (applied as qualitative parameter (yes/no)), age and body mass index (applied as quantitative parameters). Statistical calculations of logistic regression with adaptive permutation test to correct for multiple comparisons (Che et al., 2014) were performed using the PLINK package (Purcell et al., 2007). $P_{perm} \leq 0.017$ was accepted as statistically significant value (the numbers of examined genetic models was the basis for Bonferroni correction, n = 3) (Starikova et al., 2020). The Solid Spine method of linkage disequilibrium with D' > 0.80 realized in the HaploView computer program (Barrett et al., 2005) was used to infer haplotype. In evaluating the haplotype association analyses results, $p_{perm} \leq 0.05$ was adopted as statistically significant.

The interlocus epistatic interactions between the *FLG* polymorphisms in n-order models (two-, three-, and four-locus models were generated) were analyzed by the MB-MDR (Model-Based Multifactor Dimensionality Reduction) package for R (Calle et al., 2010). The significance of the interlocus interaction models was estimated by the permutation procedure (Che et al., 2014). We applied a conservative significance threshold to selected epistatic models for the permutation test based on the Bonferroni correction that considers the total numbers of combinations examined for ten loci: $p_{interaction} < 1.11 * 10^{-3}$ (<0.05/45) for the two-locus models, $p_{interaction} < 4.17 * 10^{-4}$ (<0.05/120) for the three-locus models, and $p_{interaction} < 2.38 * 10^{-4}$ (<0.05/210) for the four-locus models. A significant level for the permutation procedure was accepted at $p_{perm} < 0.001$ (Ponomarenko et al., 2021).

2.4. Identification of plausible target genes of AD risk variants

To determine functionality of the variants associated with the AD risk or their proxies SNPs ($r^2 \geq 0.8$), we utilized several bioinformatic resources available online (Moskalenko et al., 2021; Polonikov et al., 2021): SIFT (Kumar et al., 2009) and PolyPhen-2 (Adzhubei et al., 2013) databases (detect nsSNPs and discover their functional predictions), HaploReg (disclose epigenetic effects) (Ward and Kellis, 2016), GTEx Consortium data (reveal expression and alternative splicing quantitative trait loci) (GTEx Consortium, 2020), Gene Ontology resource (identify biological processes enriched amongst the AD target genes) (Carbon et al., 2021) and GeneMANIA prediction server (estimation biological network integration for AD target genes) (Warde-Farley et al., 2010). The proxy SNPs ($r^2 \geq 0.8$) were determined using HaploReg (Ward and Kellis, 2016) and the 1000 Genomes Project Phase 1 data (European population).

3. Results

Baseline and clinical characteristics of the patient and control groups are provided in Table 1. The control group was matched to the AD patients for sex, age, body mass index, and the other characteristics ($p > 0.05$).

3.1. SNP association analyses

The summary information about the analyzed loci is given in

Table 1
Baseline and clinical characteristics of the study participants.

Parameters	Control mean ± SD, % (n)	AD mean ± SD, % (n)	p
N	612	700	–
Age, years (min–max)	42.56 ± 15.42 (18–88)	42.73 ± 17.53 (19–86)	0.82
Gender ratio, f/m	70.59/29.41 (432/180)	67.71/32.29 (474/226)	0.29
BMI, kg/m2	24.52 ± 5.09	24.80 ± 5.30	0.33
Region of residence (age of onset), urban/rural area	80.23/19.77 (491/121)	76.57/23.43 (536/164)	0.12
Current smoking	11.93 (73)	13.14 (92)	0.56
Alcohol consumption	36.11 (221)	38.14 (267)	0.48
Social class*:			0.31
I/II	43.30 (265)	46.28 (324)	
III	46.08 (282)	44.43 (311)	
IV/V	10.62 (65)	9.29 (65)	
Allergic disorders (asthma, hay fever, allergic conjunctivitis, sensitization to allergens)	–	58.43 (409)	–
Family history of allergic diseases (AD, asthma, hay fever)	–	38.29 (268)	–
Age of self-reported AD onset, years	–	38.59 ± 17.76	–
AD severity (identified by EASI):	–		–
Mild		56.71 (397)	
Moderate		38.72 (271)	
Severe		4.57 (32)	

* Registrar General's social class: *I*, professional; *II*, managerial and technical; *III*, skilled; *IV*, partly skilled; and *V*, unskilled.

Supplementary Table 1. The observed allele and genotype frequencies of all polymorphisms were in Hardy-Weinberg equilibrium ($p > 0.05$). Two SNPs, rs3126085 и rs12144049, were associated with a higher risk of AD according to the allelic (for allele A OR = 1.75, 95 %Cl 1.22–2.48, p = 0.002, $p_{perm} = 0.002$ and for allele C OR = 1.45, 95 %Cl 1.08–1.93, p = 0.011, $p_{perm} = 0.011$ respectively), additive (for allele A OR = 1.69, 95 %Cl 1.20–2.38, p = 0.003, $p_{perm} = 0.004$, power = 99.63% and for allele C OR = 1.47, 95 %Cl 1.09–1.97, p = 0.011, $p_{perm} = 0.011$, power − 98.14% respectively) and dominant (OR = 1.79, 95 %Cl 1.21–2.65, p = 0.003, $p_{perm} = 0.004$, power = 99.61% and OR = 1.63, 95 %Cl 1.16–2.28, p = 0.005, $p_{perm} = 0.005$, power = 99.03%, respectively) genetic models (Table 2).

The haploblock structure of the analyzed SNPs is shown in Fig. 1. The haploblock structures in the patients and the controls were different. The former possessed a single haploblock including four SNPs, whereas the latter had three haploblocks comprising seven SNPs (Fig. 1). Five haplotypes within these haploblocks were associated with AD (Table 3). The strongest and most significant association was demonstrated by haplotype GT[rs3126085-rs12144049] (OR = 0.60, p = 0.00007, p_{perm} = 0.001). In addition, seven more loci (rs12130219, rs558269137, rs61816761, rs6661961, rs471144, rs10888499, and rs77199844) were associated with AD within>30 haplotypes (Table S2). Haplotypes GGT [rs61816761-rs3126085-rs12144049] and AWGGT[rs12130219-rs558269137-rs61816761-rs3126085-rs12144049] manifested the most significant association (OR = 0.59, p = 0.00002, $p_{perm} = 0.001$ and OR = 0.63, p = 0.00007, $p_{perm} = 0.001$, respectively).

Seven genetic variants (rs12130219, rs3126085, rs12144049, rs6661961, rs471144, rs10888499, and rs4363385) interacted within ten best n-order SNP × SNP epistatic models ($p_{perm} < 0{,}001$) to confer susceptibility to AD (Table 4). Polymorphism rs12144049 was involved in all ten best SNP × SNP interactions models, loci rs12130219 and rs3126085 contributed to seven and five models, respectively. More than 25 genotype combinations were determined within these high-

Table 2
Associations of the *FLG* gene polymorphisms with AD. All results were obtained after adjustment for covariates, *OR*, odds ratio, *95 %CI*, 95% confidence interval, p values < 0.017 are shown in bold.

SNP	Minor allele	n	Allelic model				Additive model				Dominant model				Recessive model			
			OR	95 %CI		P	OR	95 %CI		P	OR	95 %CI		P	OR	95 %CI		P
				L95	U95			L95	U95			L95	U95			L95	U95	
rs12130219	G	1290	1.06	0.82	1.38	0.617	1.06	0.82	1.37	0.645	1.19	0.87	1.63	0.282	0.70	0.36	1.35	0.288
rs558269137	delACTG	1280	1.45	0.70	3.00	0.306	1.47	0.71	3.06	0.304	1.47	0.71	3.06	0.304	–	–	–	–
rs6661961	T	1294	1.01	0.81	1.26	0.917	1.02	0.82	1.26	0.888	1.05	0.76	1.44	0.785	0.98	0.66	1.47	0.936
rs3126085	A	1282	**1.75**	**1.22**	**2.48**	**0.002**	**1.69**	**1.20**	**2.38**	**0.003**	**1.79**	**1.21**	**2.65**	**0.003**	2.48	0.78	7.89	0.123
rs12144049	C	1244	**1.45**	**1.08**	**1.93**	**0.011**	**1.47**	**1.09**	**1.97**	**0.011**	**1.63**	**1.16**	**2.28**	**0.005**	1.18	0.50	2.81	0.709
rs61816761	A	1294	5.22	0.62	43.51	0.088	5.43	0.65	45.39	0.119	5.43	0.65	45.39	0.119	–	–	–	–
rs471144	G	1276	1.04	0.67	1.59	0.863	1.04	0.69	1.59	0.842	0.98	0.62	1.55	0.927	3.52	0.39	31.84	0.263
rs10888499	C	1294	0.83	0.64	1.05	0.131	0.82	0.64	1.06	0.127	0.70	0.51	0.95	0.023	1.26	0.67	2.34	0.474
rs77199844	delAT	1286	1.01	0.63	1.59	0.972	1.01	0.62	1.62	0.982	1.01	0.62	1.62	0.982	–	–	–	–
rs4363385	T	1264	0.97	0.77	1.22	0.802	0.97	0.78	1.22	0.825	0.88	0.63	1.23	0.467	1.10	0.74	1.65	0.631

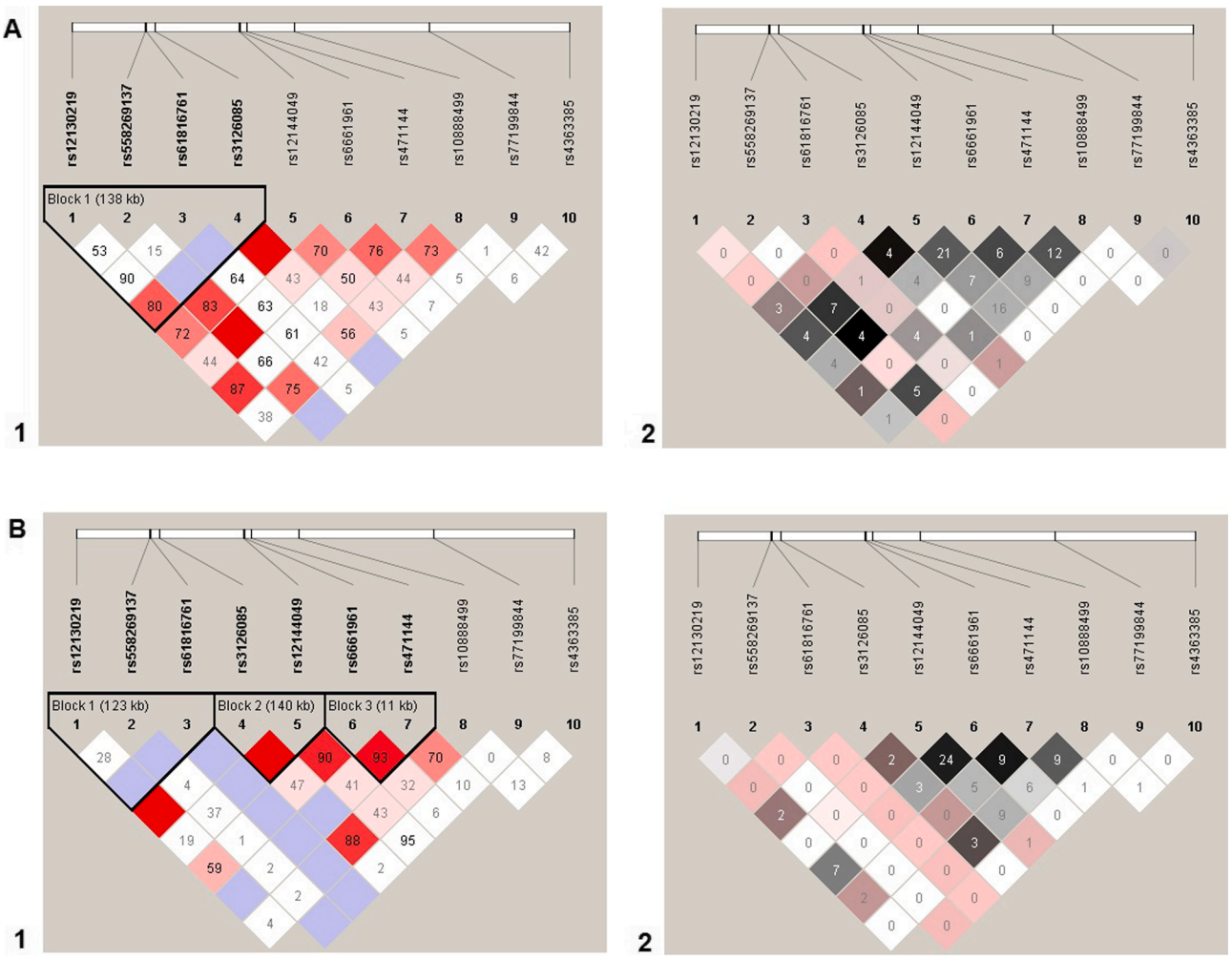

Fig. 1. Linkage disequilibrium (LD) between ten studied *FLG* gene SNPs. (A) AD patients, (B) control group. LD values are presented as Lewontin's standardized coefficient D′ (Figure sections 1) and the square of the correlation Pearson's coefficient (r^2) (Figure sections 2) between the SNPs.

Table 3
Haplotype associations of the *FLG* gene polymorphisms with AD.

Haploblocks	SNPs	Haplotypes	Frequency		OR	p	p_{perm}
			Cases	Controls			
H1	rs3126085|rs12144049	GC	0.224	0.165	1.47	0.011	0.033
	rs3126085|rs12144049	AT	0.144	0.094	1.58	0.010	0.029
	rs3126085|rs12144049	GT	0.632	0.741	0.60	0.00007	0.001
H2	rs6661961|rs471144	TG	0.063	0.063	1.00	0.991	–
	rs6661961|rs471144	TT	0.345	0.338	1.03	0.817	–
	rs6661961|rs471144	AT	0.592	0.599	0.96	0.697	–
H3	rs12130219|rs558269137|rs61816761	AdelG	0.022	0.013	1.94	0.173	–
	rs12130219|rs558269137|rs61816761	GWG	0.237	0.224	1.06	0.636	–
	rs12130219|rs558269137|rs61816761	AWG	0.741	0.763	0.87	0.296	–
H4	rs12130219|rs558269137|rs61816761|rs3126085	AWGA	0.140	0.087	1.70	0.004	0.011
	rs12130219|rs558269137|rs61816761|rs3126085	AdelGG	0.023	0.011	2.35	0.099	–
	rs12130219|rs558269137|rs61816761|rs3126085	GWGG	0.229	0.223	1.03	0.839	–
	rs12130219|rs558269137|rs61816761|rs3126085	AWGG	0.608	0.679	0.73	0.007	0.020

All results were obtained after adjustment for covariates, *OR*, odds ratio, *p*, significance level, p_{perm}, significance level after the permutation test (1000 permutations), Alleles for rs558269137 are denoted as follows: W, ACTG; del, delACTG.

order epistatic models, four of them conferred a lower risk for the disease (p = 0.000002): rs12144049 TT × rs3126085 GG (*beta* = −0.68), rs10888499 AC × rs12144049 TT (*beta* = -0.85), rs12130219 AA × rs12144049 TT × rs3126085 GG (*beta* = −0.87), rs12130219 AA × rs12144049 TT × rs471144 TT × rs3126085 GG (*beta* = −0.82) (Table S3).

The graph of the SNP × SNP interactions (Fig. 2) shows the largest contribution to the entropy (susceptibility to the disease) by both the loci independently associated with AD (1.13% by rs12144049 and 1.04% by rs3126085) and several interlocus interactions of these and other variants. The interactions were either antagonistic (e.g., −0.80% for the pair rs3126085 × rs10888499) or synergistic (e.g., 0.60% for the

Table 4
SNP × SNP interactions of the *FLG* gene loci significantly associated with AD.

N	SNP × SNP interaction models	NH	*beta*H	WH	NL	*beta*L	WL	p_{perm}
Two-order interaction models ($p < 1.2 * 10^{-4}$)								
1	rs10888499 × rs12144049	1	0.581	4.80	1.00	−0.852	18.38	< 0.001
2	rs12144049 × rs3126085	2	0.536	9.45	1.00	−0.676	18.05	< 0.001
3	rs12130219 × rs12144049	1	0.596	8.89	1.00	−0.637	14.93	< 0.001
Three-order interaction models ($p < 2.2 * 10^{-6}$)								
1	rs12130219 × rs10888499 × rs12144049	4	0.858	22.43	3.00	−0.741	21.15	< 0.001
2	rs12130219 × rs12144049 × rs3126085	3	0.676	13.50	1.00	−0.874	22.13	< 0.001
3	rs12130219 × rs6661961 × rs12144049	3	0.757	12.41	3.00	−0.831	19.89	< 0.001
4	rs10888499 × rs4363385 × rs12144049	2	1.135	8.53	2.00	−0.963	19.56	< 0.001
Four-order interaction models ($p < 1.6 * 10^{-6}$)								
1	rs12130219 × rs6661961 × rs12144049 × rs3126085	2	1.170	9.13	3.00	−1.297	29.68	< 0.001
2	rs12130219 × rs10888499 × rs12144049 × rs3126085	4	0.794	14.58	3.00	−0.894	26.43	< 0.001
3	rs12130219 × rs12144049 × rs471144 × rs3126085	1	1.151	4.04	2.00	−0.894	22.98	< 0.001

The results were obtained using the MB-MDR method with adjustment for covariates, *NH*, number of significant high risk genotypes in the interaction, *beta H*, regression coefficient for high risk exposition in the step2 analysis, *WH*, Wald statistic for high risk category, *NL*, number of significant low risk genotypes in the interaction, *beta L*, regression coefficient for low risk exposition in the step2 analysis, *WL*, Wald statistic for low risk category, p_{perm}, permutation p-value for the interaction model (1.000 permutations).

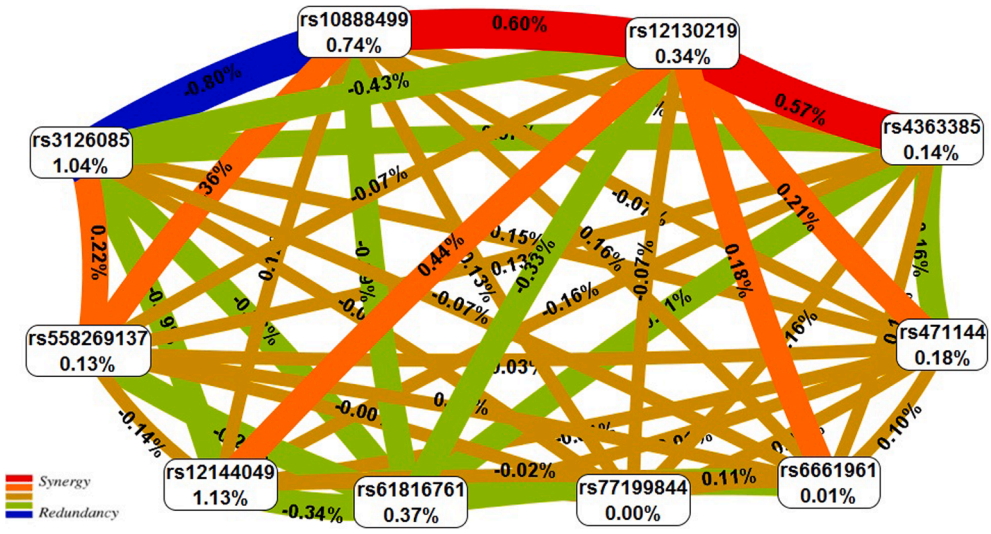

Fig. 2. The entropy graph of the SNP × SNP interactions with AD based on the MDR analysis. Positive values of entropy indicate synergistic interactions while the negative values indicate redundancy. The red and orange colors denote strong and moderate synergism, respectively, brown color denotes the independent effect, green and blue colors denote moderate and strong antagonism. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pair rs10888499 × rs12130219 and 0.57% for the pair rs12130219 × rs4363385).

3.2. Functional SNPs

Non-synonymous SNPs. Among the AD-associated SNPs, two polymorphisms are loss-of-function variants: rs61816761 (R501X) is a nonsense mutation and rs558269137 (2282delACTG) is a frameshift variant. Locus rs3126085 was in strong LD with 21 nsSNPs, twelve of those had pronounced prediction effects denoted as «deleterious», «probably damaging», and «possibly damaging» (Table S4).

Regulatory effects. The characteristics of the epigenetic effects of the AD-associated loci and 136 proxy SNPs are shown in Table S5. According to the HaploReg database, these loci were located in the various regions, such as exons, introns, 3′-UTR, and 5′-UTR of sixteen genes (*FLG, FLG2, FLG-AS1, CRCT1, CRNN, HRNR, LCE1E, LCE3E, LCE5A, RP1-91G5.3, SNORA31, SPRR1A, SPRR1B, SPRR2B, SPRR2D,* and *SPRR3*).

SNPs in LD with the AD risk variants showed considerable regulatory effects. For example, among 81 variants linked to rs3126085 (demonstrated the main effects on AD risk), 70 loci were designated to produce epigenetic effects. The AD-associated loci and their proxies demonstrated their functional effects in multiple cell types (e.g., hESC derived CD56 + ectoderm cultured cells, H1 and H9 derived neuronal progenitor cultured cells, brain, blood, etc.) related to the AD pathophysiology, including cultures of skin cell (NHEK-epidermal keratinocyte primary cells, NHDF-ad adult dermal fibroblast primary cells), which are target tissues for AD.

Expression QTLs. The data from the GTEx consortium suggests that nine of the ten AD-associated loci are eQTLs (Table S6) determining transcription of 33 target genes in many tissue/organ types, including the skin (Table S7). We found that 116 SNPs in LD with the AD loci were also significantly correlated with mRNA levels of 23 genes in multiple tissues/organs (Table S8), including 12 genes in the skin (Table S9). In summary, the AD-associated loci of the *FLG* gene and their proxies apparently affect the transcription levels of 34 genes including seventeen skin-specific genes (*CRCT1, CRNN, FLG, FLG2, FLG-AS1, HRNR, LCE1D, LCE1E, LCE3A, LCE3C, LINGO4, RP1-91G5.3, SPRR1A, SPRR1B, SPRR2B, SPRR2D,* and *SPRR3*). Interestingly, the AD risk allele A rs3126085 is associated with the lower expression of the *FLG* gene in the skin ($\beta = -0.22$, $p = 3.7 * 10^{-8}$, $p_{FDR} \leq 0.05$).

Splicing QTLs. We detected significant SNP-gene splicing associations: two SNPs (rs6661961 and rs4363385) were associated with sQTL independently, and three SNPs (rs3126085, rs6661961, and rs4363385) were linked to 28 loci affecting alternative splicing of four genes (*CRNN, SPRR3, RP1-20N18.10,* and *RP11-107M16.2*) (Table S10).

3.3. Identify biological processes enriched amongst the AD target genes

Two strategies were used to identify biological processes underlying the observed associations. First, we considered roles in biological processes or molecular functions of genes likely affected by the functional effects of the AD risk variants and their proxies in various tissue/organs – 38 genes in total (Table S5; Table S6; Table S8; Table S10). Second, we tested a significant pathway enrichment only for skin-specific genes, i.e., showing expression and epigenetic effects in the skin – 20 genes in total (Table S5; Table S7; Table S9).

The biological process enrichment analysis of the abovementioned 38 genes suggested that they influence formation of the cornified envelope (FDR = 2.52E-21), keratinocyte differentiation (FDR = 4.52E-18), keratinization (FDR = 6.31E-19), cornification (FDR = 3.26E-10), and more than twenty other pathways related to skin development, programmed cell death, regulation of water loss via the skin, etc. (Table S11). The interaction network of the above genes and other genes is shown in Fig. 3. These interactions are realized mainly through common protein domains (52.36%), co-expression (41.79%), and co-localization (3.87%) (Table S12).

The biological processes enriched for the 20 skin-specific target genes (Table S13) are quite similar to those for the 38 AD risk genes (Table S11). According to the network shown in Fig. 4, the interactions between the 20 skin-specific genes and the other genes are executed through common protein domains (58.07%), co-expression (35.68%), and co-localization (6.25%). The AD-associated genes may interact either directly or via other genes (e.g., *CRCT1, SPRR1A, SPRR3,* etc.) (Table S14).

4. Discussion

In the present study, we replicated the association of ten SNPs of the *FLG* gene with AD in Caucasians from the central region of Russia: two SNPs demonstrated an independent association, nine SNPs were associated within 30 haplotypes, and seven SNPs manifested significant interactions within ten interlocus epistatic models. Alleles A rs3126085 and C rs12144049 were individually associated with an increased risk of AD (OR = 1.69–1.79 and 1.45–1.63, respectively). The AD protective haplotypes GT[rs3126085-rs12144049] (OR = 0.60), GGT[rs61816761-rs3126085-rs12144049] (OR = 0.59), and AWGGT[rs12130219-rs558269137-rs61816761-rs3126085-rs12144049] (OR = 0.63) had the most significant association (p_{perm} = 0.001).

The *FLG* gene is located on chromosome 1q21.3 in a region called the epidermal differentiation complex and encodes filaggrin, a major structural protein in the stratum corneum (Kim et al., 2019). The protein is a key component of the natural moisturizing factors important for epidermal water retention and low acidity of the outermost stratum corneum (Kabashima, 2013). Filaggrin plays a role in the differentiation of keratinocytes and the maintenance of epidermal integrity. Decreased production of FLG metabolites results in elevated skin surface pH or activating neutral pH-dependent kallikreins that affect skin barrier function (Al-Shobaili et al., 2016). Defects in FLG and other epidermal barrier proteins result in uncontrolled immune responses to external antigens and induce skin and systemic inflammatory responses (Kabashima, 2013). Genetically determined FLG insufficiency (e.g., due to the *FLG* null mutations, etc.), epigenetic mechanisms (DNA methylation, non-coding RNAs, etc.), environmental factors (*Staphylococcus aureus* skin colonization, etc.)) are among the key risk factors of AD (Kabashima, 2013; Al-Shobaili et al., 2016; Ng and Chew, 2020). *FLG* is significantly downregulated in the skin of AD patients (Pellerin et al., 2013).

The observed associations of alleles A rs3126085 and C rs12144049 correspond to the results of the earlier GWAS (Sun et al., 2011; Baurecht et al., 2015; Schaarschmidt et al., 2015). Specifically, Sun et al. (2011)

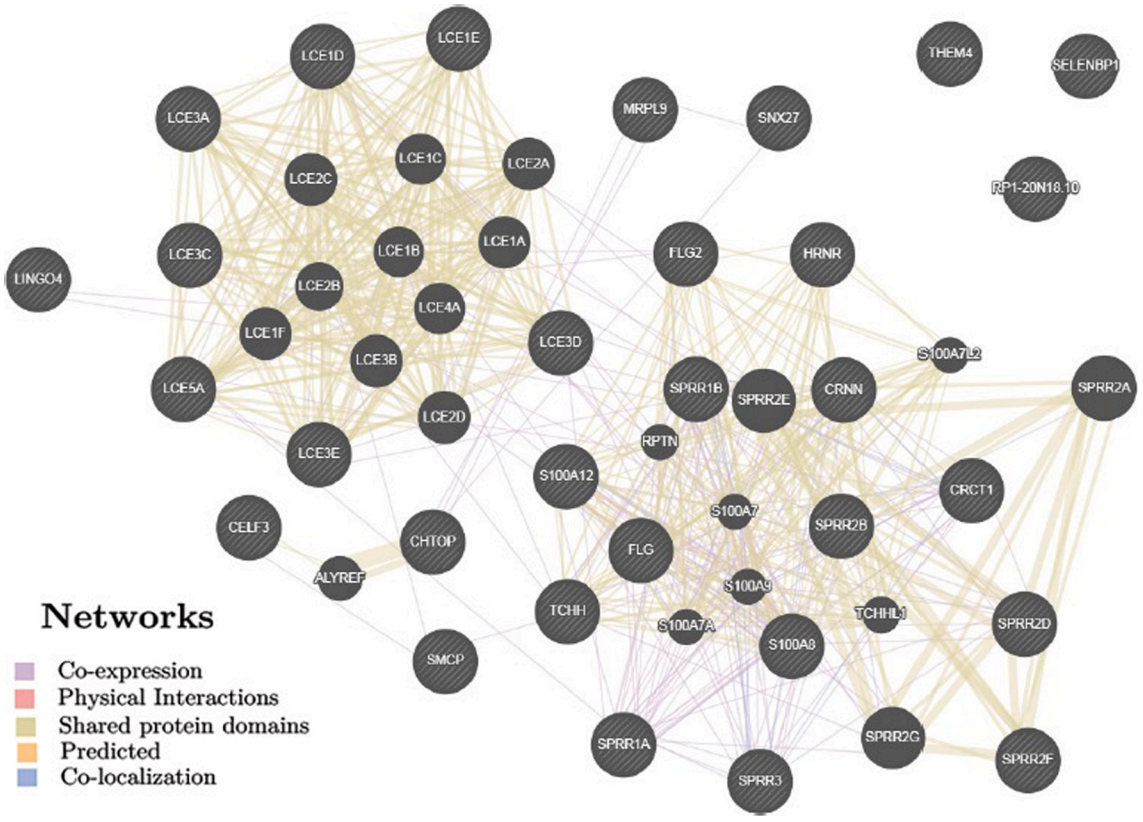

Fig. 3. The interaction networks of the AD candidate genes in various tissues/organs inferred using GeneMANIA (http://genemania.org). The candidate genes are cross-shaded.

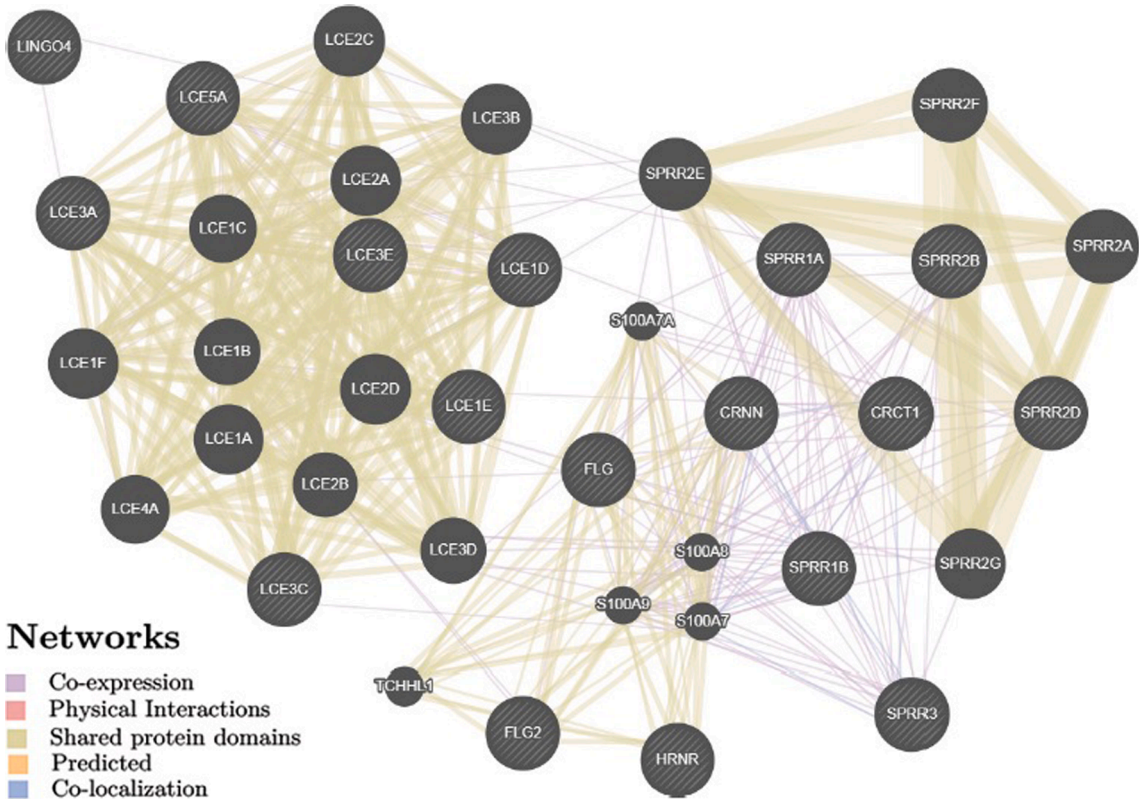

Fig. 4. The interaction networks of the skin-specific AD risk candidate genes according to GeneMANIA (http://genemania.org). The candidate genes are cross-shaded.

reported a protective effect for AD of the minor allele G rs3126085 in the Chinese population (OR = 0.82). The authors suggested that risk allele A rs3126085 was apparently correlated with the c.3321delA variant (the most common mutation of *FLG* in the Chinese population) and that the association of rs3126085 with AD was mainly driven by the c.3321delA mutation. On the other hand, a replicative analysis conducted on the German sample in that study did not find any association of the rs3126085 variant with AD. On the contrary, such an association was demonstrated in another study of a German population (Schaarschmidt et al., 2015). Worth mentioning that in the analyzed Caucasian populations (German in the above studies and Russian in the present study), a minor allele of rs3126085 is A, whereas in the Chinese population it is G. European AD risk allele C rs12144049 previously reported by two GWAS (Baurecht et al., 2015; Schaarschmidt et al., 2015) was confirmed in the present study too.

Six *FLG* gene polymorphisms (rs12130219, rs12144049, rs471144, rs10888499, rs77199844, rs4363385) out of the ten analyzed in the present study were previously reported for their association with the disease (Baurecht et al., 2015). Two of them (rs12130219, rs4363385) were also associated with psoriasis. Interestingly, this study identified risk alleles with the opposing effect for AD и psoriasis at both shared and independent disease-specific loci within the epidermal differentiation complex (chromosome 1q21.3). For example, the G allele of rs12130219 decreases the risk for AD (OR_{ADcond} = 0.81) and increases the risk for psoriasis ($OR_{PSOcond}$ = 1.12). A similar opposing effect for AD and psoriasis was documented for rs4363385 too. The authors suggested that genetic factors determining keratinocyte differentiation and cutaneous barrier function had the strongest effects on the AD risk (that was also supported by the results of the present study), whereas genetic factors affecting (auto-)antigen recognition are the most important for the psoriasis risk. Besides, the results of the Gene Ontology enrichment pathway analysis by Baurecht H. et al. (2015) suggested process "keratinocyte differentiation" (GO:0030216) (FDR = 4.3×10^{-4}) as a significant contributor to AD. This finding was supported by the results of the present study with an even higher significance (FDR = 4.52E-18 for all AD-associated genes and FDR = 5.35E-18 for the skin-specific genes).

The present study suggests that ten AD risk variants and their 136 proxy SNPs may contribute to the risk of AD through various functional effects. These genes are similar in structure and function and are located near each other in the epidermal differentiation complex (Al-Shobaili et al., 2016). Several of them, e.g., *FLG*, *HRNR* (hornerin), *CRNN* (cornulin), *S100A8*, S100A12, encode proteins of the S100 fused-type family contributing to the cornified cell envelope, a functional component of the epidermal barrier (Baurecht et al., 2015). The contribution of polymorphisms in the region of the *FLG-AS1/RPTN/HRNR* genes, *SPRR* and *LCE3* clusters, etc. (1q21.3) was suggested by several GWAS (Sun et al., 2011; Baurecht et al., 2015; Ellinghaus et al., 2013, etc.). The SNPs located on 1q21 in/near *HRNR, FLG*, and *SPRR2A* were reported to affect age of eczema onset (Ferreira et al., 2020). The expression level of cornified envelope proteins in the AD skin is significantly different as compared to the healthy controls: *FLG, FLG2, LOR, CRNN*, and *SPRR3-v1* are downregulated, whereas *RPTN, HRNR*, and *SPRR1Av1* are upregulated (Trzeciak et al., 2020). Importantly, the *in silico* analysis of the present study also showed the association of the AD risk allele A rs3126085 with the lower expression of the *FLG* gene in the skin. The function of *FLG-AS1* (*FLG* antisense RNA1) is currently unknown, but its proximity to *FLG* and *HRNR* suggests a role in keratinocyte differentiation (Baurecht et al., 2015). In the AD lesional skin, the *FLG-AS1* expression is reduced (Baurecht et al., 2015). The *FLG2* gene encodes a histidine- and glutamine-rich protein, which shares common structural features with other SFTP members, in particular filaggrin (Wu et al., 2009). Functions of FLG2 and filaggrin in the formation of the epidermal barrier may overlap and even be synergistic, protecting the skin from unfavorable environmental influences and water loss by generating precursors of natural moisturizing factors (Wu et al., 2009).

The AD-associated loci of the *FLG* gene determined in the present study appear to be important not only for skin diseases (e.g., AD, psoriasis, etc.) (Sun et al., 2011; Weidinger et al., 2013; Baurecht et al., 2015; Schaarschmidt et al., 2015, present study) but also for some allergic disorders (e.g., asthma, hay fever) (Ferreira et al., 2017; Ferreira et al., 2019; Zhu et al., 2019; Olafsdottir et al., 2020).

Some limitations of this study should be acknowledged though. In particular, the current sample size of the AD patients is not large enough to provide sufficient power for the association analysis of the *filaggrin* gene polymorphisms with clinical characteristics. The planned two-fold increase in the patient cohort sample size will make the proposed analysis (a genotype-phenotype correlation) more feasible.

5. Conclusions

The *FLG* gene polymorphisms were associated with AD in Caucasians of the Central region of Russia. Thirty-eight genes in various tissues/organs (including 20 genes in the skin) were apparently affected by various functional effects (epigenetic, eQTL, sQTL, and non-synonymous) of the ten AD risk variants and their proxies. The biological process enrichment analyses suggested that the plausible AD candidate genes influence the formation of the cornified envelope, keratinization, cornification, and other more twenty pathways related to skin development, programmed cell death, regulation of water loss via the skin.

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CRediT authorship contribution statement

Mikhail Churnosov: Project administration, Funding acquisition, Formal analysis, Visualization, Supervision, Methodology, Writing – original draft. **Tatyana Belyaeva:** Methodology, Formal analysis, Supervision, Writing – review & editing. **Evgeny Reshetnikov:** Methodology, Formal analysis, Writing – review & editing. **Volodymyr Dvornyk:** Data curation, Methodology, Formal analysis, Writing – review & editing, Supervision, Methodology. **Irina Ponomarenko:** Conceptualization, Methodology, Validation, Formal analysis, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2022.146219.

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