FILAGGRIN LOSS-OF-FUNCTION MUTATIONS 2282DEL4, R501X, R2447X AND S3247X IN ATOPIC DERMATITIS

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Atopic dermatitis (AD) is a widespread multifactorial genetically determined inflammatory skin disease caused by, among other causes, impaired functions of the epidermal barrier. Loss-of-function mutations of the filaggrin gene (important part of the natural moisturizing factor system) that arrest production of the full-fledged precursor protein is associated with AD. This study investigated the frequency of the 2282delACTG (rs558269137), R501X (rs61816761), S3247X (rs150597413), R2447X (rs138726443) loss-of-function mutations of the filaggrin gene in adults of European origin with moderate to severe AD. The study involved 99 adult patients of both sexes aged 18-68 years. The mutations were identified with the help of the purpose-developed method of multiplex analysis of four single nucleotide polymorphisms that relies on the SNaPshot technique (minisequencing). The incidence of loss-of-function mutation of filaggrin 2282delACTG was 5.3%, that of R501X — 0.5%, R2447X — 1%. No S3247X mutation was detected in the sample. Collation of the results with Russian and European samples revealed a comparable level of the analyzed filaggrin gene mutations in adult patients with AD from different regions of the Russian Federation.

Keywords: atopic eczema, filaggrin, loss-of-function mutation, SNaPshot technique, SNP

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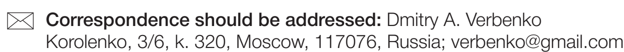
Compliance with ethical standards: the study was approved by the Ethics Committee of the State Research Center of Dermatovenereology and Cosmetology (Minutes #1 of January 29, 2021), and meets the standards of good clinical practice and evidence-based medicine. All patients included in the study have read and signed a voluntary informed consent to participate therein.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МУТАЦИИ 2282DEL4, R501X, R2447X, S3247X ФИЛАГГРИНА ПРИ АТОПИЧЕСКОМ ДЕРМАТИТЕ

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Атопический дерматит (АтД) — широко распространенное мультифакторное генетически детерминированное воспалительное заболевание кожи, обусловленное среди прочих причин нарушением функций эпидермального барьера. Нулевые мутации гена филаггрина — важного компонента системы натурального увлажняющего фактора, приводящие к отсутствию выработки полноценного белка-предшественника, ассоциированы с АтД. Цель исследования — оценить частоту наиболее распространенных в европейских популяциях нулевых мутаций гена филаггрина 2282delACTG (rs558269137), R501X (rs61816761), S3247X (rs150597413), R2447X (rs138726443) у взрослых пациентов со среднетяжелой и тяжелой степенью АтД. Анализ проведен у 99 взрослых пациентов обоих полов в возрасте 18–68 лет, со среднетяжелым и тяжелым АтД. Идентификацию мутаций осуществляли с помощью разработанного метода мультиплексного анализа четырех однонуклеотидных полиморфизмов при использовании минисеквенирования. Частота встречаемости нулевой мутации филаггрина 2282delACTG оказалась на уровне 5,3%, R501X — на уровне 0,5%, R2447X — на уровне 1%. Нулевая мутация S3247X гена *FLG* в выборке пациентов не обнаружена. Сравнение результатов с российскими и европейскими выборками выявило сопоставимый уровень анализируемых мутаций гена филаггрина у взрослых пациентов с АтД из различных регионов Российской Федерации.

Ключевые слова: атопический дерматит, филаггрин, нулевые мутации, минисеквенирование, однонуклеотидный полиморфизм

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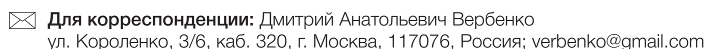
Вклад авторов: А. Э. Карамова, В. В. Чикин, К. М. Аулова, П. В. Городничев — обследование пациентов, постановка диагноза, расчет клинического индекса SCORAD, получение информированного согласия и забор биоматериала пациентов; Д. А. Вербенко — планирование исследования, выполнение молекулярно-генетических экспериментов, написание рукописи; И. В. Козлова — анализ уникальности гибридизующихся к нулевым мутациям олигонуклеотидов в гене *FLG*; А. Э. Карамова — редактирование рукописи; А. А. Кубанов — общее руководство, редактирование рукописи.

Соблюдение этических стандартов: исследование одобрено этическим комитетом при ФГБУ «Государственный научный центр дерматовенерологии и косметологии» (протокол заседания № 1 от 29 января 2021 г.), соответствует стандартам добросовестной клинической практики и доказательной медицины. Все включенные в исследование пациенты ознакомились и подписали добровольное информированное согласие на участие в его проведении.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Atopic dermatitis (AD) is a multifactorial genetically determined, chronic and recurrent inflammatory skin disease characterized by itching, age-dependent localizations and morphology of lesions. This is one of the most common skin diseases [1–2]. The prevalence and incidence of AD in the entire population of the Russian Federation (RF) in 2022 amounted to 384.7 and 157.1 cases per 100,000 population, respectively [3, 4]. In German-speaking countries, 25% of all referrals to a dermatovenerologist turn out to have moderate or severe AD [5].

A filaggrin gene mutation and change of function of the epidermal barrier are the genetic factors of AD development [2]. Hereditary determination plays an important role in its the pathogenesis, causing skin barrier disruptions, immunity defects (stimulation of Th2 cells with subsequent hyperproduction of IgE), hypersensitivity to allergens and non-specific stimuli, colonization by pathogenic microorganisms (*Staphylococcus aureus*, *Malassezia furfur*), and autonomic nervous system disbalance with increased production of inflammatory mediators [1, 6, 7]. Twin and family studies confirm the magnitude of effect of hereditary factors (72–90%) on the development of the disease. Eighty percent of children whose parents have AD suffer from the disease; this figure goes down to 50% if only one parent has AD, but the risk grows 1.5-fold if that parent is mother [8]. Thus, genes encoding structural and functional proteins of the epidermis and those managing innate and acquired immunity underly pathogenesis of the disease [1, 9, 10]. However, genome-wide association studies (GWAS) show that genomic regions associated with AD explain only about 15% of cases of the disease, and the remaining variability, the so-called "missing heritability" peculiar to complex diseases, may stem from its pronounced heterogeneity, cumulative effects of structural variations, and fluctuations of quantity of genomic copies, as well as epigenetic influences [8-10].

The size of the *FLG* gene is 2 kb; it is located on the 1st chromosome of the epidermal differentiation complex counting 70 genes [1, 11]. The gene consists of three exons, the first two of which regulate its expression, and the third contains from 10 to 13 tandemly arranged filaggrin repeats [12]. The product of the gene is profilaggrin, a protein expressed in the epidermis basal cells. Diffused into the upper layers of the outer skin, it undergoes a number of biochemical transformations that result in formation of filaggrin monomers, degradation of which produces components that are part of the natural moisturizing factor [8, 13].

Numerous studies have confirmed that loss-of-function mutations in the filaggrin gene, which stop production of the protein, are the key AD development factor [1, 2, 8, 11–18]. Carriers of such mutations run a 3.12-fold greater risk of having this disease, and in the Russian population, the ultimate risk may be 8.13 times greater [19, 20]. In the AD pathogenesis, *FLG* gene loss-of-function mutations compromise protective function of the skin barrier, which facilitates penetration of the epidermis by environmental factors that initiate inflammatory reactions and subsequent moderate-to-severe course of the disease.

The frequency of such mutations has been shown to be increased in AD patients, and their spectrum revealed to depend on these patients' ethnicity [15]. In European populations, the most common loss-of-function mutations are 2282del4, R501X, R2447X, S3247X, and in Asian populations - c.3321delA, c.6950del8, p.S2706X, p.K4022X, p.E2422X, p.Q2417X, p.S1515X, p.S406X, etc. [11–18]. There is evidence of a correlation between the frequency of *FLG* gene loss-of-function mutations and the severity of AD [16, 21].

The classic approach to identification of the *FLG* gene loss-of-function mutations involves a PCR with the considered locus and a subsequent restriction analysis of the product of this reaction [22]. However, unstable performance of the restriction enzyme may produce a biased estimate of the frequency. Sanger sequencing of the *FLG* gene's third exon is complicated by repetitive sequences of filaggrin monomers [8, 11, 13]; the same reason is likely to be behind lack of common application of allele-specific hybridization as well as TaqMan real-time PCR [23, 24], which is also used to detect mutations of the filaggrin gene. Next-generation sequencing (NGS) can reveal the full spectrum of the *FLG* gene loss-of-function mutations [17]. However, NGS requires special equipment and large samples, since some loss-of-function mutations are rare; in addition, targeted filaggrin LOF sequencing kits are not yet commercially available in Russia. At the same time it is possible to set up single-tube studies (in any PCR laboratory equipped for fragment analysis) and identify such *FLG* gene mutations most common in the European populations.

This study aimed to estimate the frequency of 2282delACTG (rs558269137), R501X (rs61816761), S3247X (rs150597413), R2447X (rs138726443), most common *FLG* gene loss-of-function mutations in the European populations, using the new method based SNaPshot technique.

METHODS

The study was conducted in the State Research Center of Dermatovenereology and Cosmetology of the Ministry of Health of the Russian Federation. The study's inclusion and exclusion criteria have been described earlier [25].

The total number of AD patients participating in the study was 99, 50 female and 49 male, aged 18-68 years. The mean age of the patients was 31.07 ± 10.53 years; all of them have been diagnosed with AD before the study, and when included, had the disease confirmed as exhibiting features described by J.M. Hanifin and G. Rajka [2, 26]. Ninety-six patients were of European origin, three — Asian ethnics.

Table 1 gives the age of onset of AD in the participants.

The disease manifested primarily in childhood. Most often (49.5% of cases), the onset was registered in infancy, before the age of 1 year. Sixty-eight patients (68.7%) developed AD before the age of 3. In 88 patients, the disease first manifested when they were under 7 years of age. However, 5 (5.0%) patients reported the onset of AD in adulthood.

Allergic rhinitis was diagnosed in 17 (17.2%) patients, bronchial asthma in 15 (15.2%), pollinosis in 11 (11.1%), allergic conjunctivitis in 9 (9.1%).

The analysis of the family history considered blood-related siblings, parents and grandparents, and uncles and aunts; in 44 patients, it revealed atopic and allergic burdens. AD was diagnosed in relatives of 40 (40.4%) participants: fathers of 14, mothers of 13, grandparents of 6, siblings of 6, uncles/aunts of 7. Relatives of 8 (8.1%) AD patients suffered from bronchial asthma. Three patients reported that their fathers have been diagnosed with bronchial asthma, 2 patients pointed to this disease in their brothers, and 1 participant stated that this diagnosis was announced to his mother, grandfather and grandmother. Two (2.0%) patients mentioned relatives with pollinosis, a father of one and a grandmother of another. As for allergic rhinitis, this disorder affected both parents of one patient and father of another patient, which means there were 2 (2.0%) participants with it in their family medical history.

The severity of AD was established with the help of the SCORAD. The participants that who 25 through 50 points on its scale were considered to have moderate AD, those with the score above 50 — severe AD [27].

Table 1. Participating AD patients by the disease onset age

		Infancy (1 month to 1 year)	Early childhood (1 to 3 years)	Preschool age (3 to 7 years)	Junior and senior school age (7 to 18 years)	Early adulthood (18 to 44 years)	Total
Number of patients	абс.	49	19	20	6	5	99
	%	49,5	19,2	20,2	6,1	5	100

Thus, 64 (64.6%) patients were diagnosed with moderate AD, and 35 (35.4%) with severe atopic dermatitis. The SCORAD scores in the sample ranged from 25.2 to 77.1 points (mean — 47.20 ± 12.57 points).

The participants donated venous blood samples, 4–5 ml of which were collected into VACUETTE® K3E K3EDTA tubes (Greiner Bio-One; Austria), and separated into cellular and plasma fractions at 3000 g for 10 minutes in an Allegra X-14 centrifuge (Beckman Coulter; USA), the results of separation then stored at -20 °C until DNA extraction.

Genomic DNA was isolated from cellular biomass with the help of QIAamp DNA Mini Kit (QIAGEN; Germany), as prescribed by the manufacturer. Resulting genomic DNA's concentration and purity were analyzed in a NanoVue 2000 spectrophotometer (GeneralElectric; France).

To detect loss-of-function mutations of the FLG gene, we carried out a two-stage multiplex PCR with intermediate purification and subsequent identification of single-nucleotide polymorphisms after separation in 3130 Genetic Analyzer according to the instructions of the manufacturer of the SNaPshot kit (Applied Biosystems; USA). The sequences of oligonucleotides and hybridization probes were selected based on the data from BLAST (USA) [28], using Ugene software v.44.0 (http://ugene.net /); the synthesis was performed by Syntol LLC (Russia).

The first stage of PCR involved four regions of the *FLG* gene and was done with primers; Table 2 shows their nucleotide sequence. PCR was followed by visualization of the successful reaction in VersaDoc gel documentation system (Bio-Rad; USA), with ethidium bromide staining after separation of fragments by electrophoresis in 2% agarose gel (TAE buffer, voltage — 180 V, time — 30 minutes). To determine the molecular weight of the amplified fragments, we used a 100-1000 bps lengths marker. (Thermo Fisher Scientfic; США).

For PCR, we used in 0.1 ml tubes (Biologix; China); the volume of the mixture was 20 µl, including 10 µl of PCR buffer from the QUAGEN Multiplex PCR Kit (Germany), 5 pM of each primer per 1 µl of the mixture, from 1 to 100 nM of DNA, and deionized water. The amplification program involved DNA melting and activation of Taq polymerase for 15 minutes at 95 °C, followed by 40 cycles that included annealing of primers at 57 °C for a minute, a minute long elongation at 72 °C, and melting at 95 °C for 30 seconds. When the reaction was over, we purified the PCR products by incubation at 37 °C for 45 minutes in the presence of exonuclease I and alkaline phosphatase enzymes: 0.5 µl of ExoI and 1 µl of FastUP (Thermo Fisher Scientific; USA) were added to 5 µl of the PCR product, and 15-minute enzymes inactivation at 85 °C.

The purified PCR products from the first stage were used in the hybridization PCR with oligonucleotides flanking single nucleotide polymorphisms, the sequences of which are shown in Table 3. The reaction occurred in 0.1 ml tubes (Biologix; China), the substance volume was 10 µl, including 5 µl of the finished mixture of 2× SNaPshot PCR ready Master Mix, 3 µl of water, 1 µl of mixed purified PCR products, and 1 µl of a primers mixture. At this stage, the amplification program included 25 cycles: primers annealing at 50 °C for 5 seconds, elongation at 60 °C for 30 seconds, and melting at 95 °C for 10 seconds. Once the reaction was over, PCR products were purified through incubation at 37 °C for 45 minutes in the presence of alkaline phosphatase: 1 µl of FastUP enzyme (Thermo Fisher Scientific; USA) was added to 5 µl of the PCR product, and subsequent inactivation at 85 °C for 15 minutes.

To 0.5 µl of the purified second-stage PCR product, we added 9 µl of deionized formamide and Gene Mapper LIZ-120 molecular weight marker (Applied Biosystems; USA), denatured the resulting mixture at 95 °C for 5 minutes, then cooled it on ice.

The mixture of products of the second stage of multiplex PCR was separated in an ABI 3130 Genetic Analyser (Applied Biosystems; USA), according to the SNaPshot kit protocol. For this operation, we used a standard 50 cm capillary tube filled

Table 2. Oligonucleotide sequences of primers for multiplex PCR of target regions of the *FLG* gene

Genome region	Primer direction	Nucleotide sequence 5'–3'
2282delACTG	direct	TGGTAGTCAGGCCACTGACAGTG
	reverse	GGTGACCAGCCTGTCCATGG
R501X	direct	GACCTATTTACCGATTGCTCGTGG
	reverse	GGACGTTCAGGGTCTTCCCTCT
S3247X	direct	ACTGGACCCCCAGTGTCTACT
	reverse	GGTGGTCTGGGTCTGCTTCCAG
R2447X	direct	TGGGATGTGGTGTGGCTGTGATGAG
	reverse	CAAGGATCCCACCACAAGCAGGCA

Table 3. Detection of the *FLG* gene loss-of-function mutations 2282delACTG (rs558269137), R501X (rs61816761), S3247X (rs150597413), R2447X (rs138726443); oligonucleotide sequences of primers, second stage (hybridization PCR).

Mutation	Hybridizing primer 5'-3' nucleotide sequence	Amplicon size	Wild type alleleic
2282delACTG (rs558269137)	$(CT)_{22}$ACCAGCCTGTCCATGGCCTGACACTG	71	A
R501X (rs61816761)	$(CT)_{20}$CGCTGAATGCCTGGAGCTGTCTC	64	G
S3247X (rs150597413)	$(TC)_{11}$TGGTGTCTGGAGCCGTGCCTT	44	G
R2447X (rs138726443)	$(CT)_{13}$CCGTTGAGTGCCTGGAGCTGTCTC	51	G

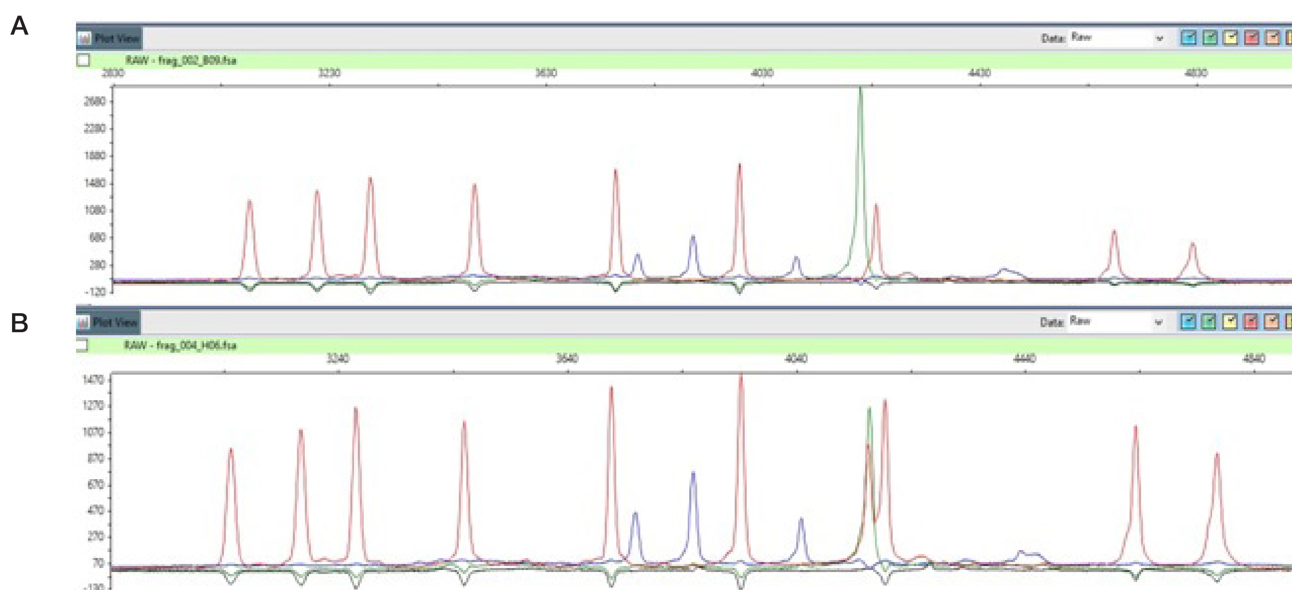

Fig. Electrophoregram of detection of the four single nucleotide polymorphisms of *FLG* gene loss-of-function mutations (minisequencing). Peak color signals the allelic variant, its size (retention time) indicates the specific polymorphism. Left to right: S3247X (rs150597413), R2447X (rs138726443), R501X (rs61816761), 2282delACTG (rs558269137). **A**. All allelic variants are wild-type, loss-of-function mutations not detected. **B**. An example of a 2282delACTG mutation heterozygote

with POP-7 polymer. The sample was introduced in the course of 7 seconds. We visualized the resulting chromatograms in Peak Scanner Software 2 (Applied Biosystems; USA), and decoded by comparing the retention time of the amplified fragments and Gene Mapper LIZ-120. The SNaPshot kit manufacturer (Applied Biosystems; USA) also offers Gene Mapper Software that can automatically decode single nucleotide polymorphisms. Comparison of the resulting data with wild-type alleles (enabled by electrophoregram imaging) allowed detecting presence of the filaggrin gene loss-of-function mutations against identification of the wild-type alleles.

To confirm detection of the 2282delACTG loss-of-function mutation by minisequencing, we analyzed an amplified fragment of the filaggrin gene (Table 1) using Ade I (Dra III) restrictase (SibEnzyme; Russia), as described in a previously published study [22]. 2282delACTG deletion creates a Dra III restrictase recognition site, which is visualized as two bands after separation of the hydrolysis products in an agarose gel.

For PCR, we used T100 (Bio-Rad; USA) and 96-well plates (Applied Biosystems; USA). The reaction was carried out in 10 μl of a mixture comprising 0.1 μm of each primer per 1 μl, 5 μl of the PCR reaction mixture from a commercially available Multiplex PCR mix kit (QIAGEN; Germany), deionized water, and a DNA matrix. The amplification program included DNA melting and polymerase activation for 15 minutes and subsequent 40 cycles of 30-second primer annealing at 54 °C, 30-second elognation, and mixture melting at 95 °C for 15 seconds.

The hydrolysis of the PCR fragment was enabled by T100 (Bio-Rad; USA) in 96-well plates (Applied Biosystems; USA); the procedure lasted for 3 hours at 37 °C, and involved 20 μl of the reaction mixture containing 5 units of restriction endonuclease, 2 μl of restriction buffer, 10 μl of the PCR product, and deionized water. The restriction sites were detected after 30-minute separation in a chamber (Bio-Rad; USA) on 2% agarose gel at 180 V by visualization of ethidium bromide-stained fragments in UV light.

RESULTS

To estimate the frequency of the filaggrin gene loss-of-function mutations 2282delACTG (rs558269137), R501X (rs61816761), S3247X (rs150597413), R2447X (rs138726443) in adult patients with moderate to severe AD, we developed a method based the detection of single-nucleotide polymorphisms that signal presence of such mutations. This method allows simultaneous identification of four *FLG* gene loss-of-function mutations; it is based on two consecutive PCRs, with the first reaction yielding the specific mutation locus, and second effecting single nucleotide elongation of the hybridizing primer with a special kit that allows determining allelic variants of a single nucleotide polymorphism. Simultaneous detection of the four considered single nucleotide polymorphisms is possible after separation in the capillary tube of 3130 Genetic Analyzer, performed as prescribed by the SNaPshot kit manufacturer (Applied Biosystems; USA); presence of the alleles different from the wild type allow conclusions about presence of loss-of-function mutations. Figure shows an example of the analysis of four loss-of-function mutations in the filaggrin gene.

The developed method of detection of loss-of-function mutations was verified by restriction analysis targeted at 2282delACTG in exon 3 of the filaggrin gene. The frequency of such mutations yielded by the analysis was 5.2%, which is comparable to the minisequencing data. Thus, the developed method of detection of the *FLG* gene loss-of-function mutations using minisequencing can be used to assess the prevalence thereof, including among the AD patients.

The study involved 99 adult patients with moderate to severe AD. In this sample, 6.8% of the participants had 2282delACTG, R501X, R2447X mutations, namely, 2282delACTG was detected in 5.05% of them, R501X — in 0.5%, R2447X — in 1%, and S3247X was not detected in any of the participating patients (Table 4). The loss-of-function mutation as a deletion of 4 p.o. in the heterozygous 2282delACTG was detected in 4% of patients, in the homozygous state — in one patient (1%), and along with R501X — in one patient, as a compound heterozygote.

DISCUSSION

The prevalence of the considered four mutations in the conditionally healthy populations represented in the 1000 Genomes catalog is below 1% [36]. The analysis of data describing adult AD patients revealed a slightly increased

Table 4. Frequency of occurrence of 2282delACTG, R501X, S3247X, R2447X in AD patients, Russian and European populations

	Sample size	2282delACTG	R501X	S3247X	R2447X	Reference
Patients with moderate to severe AD	99	5.05	0.5	0	1	
Residents of Novosibirsk	470	6.7	1.2	–	–	[29]
Russians of the central region of Russia	474	2.9	0.9	–	–	[30]
Russians of Bashkortostan	177	6.03	–	–	–	[31]
Tatars	126	9.35	–	–	–	[31]
Austrians and Germans	462	14.7	6.5	3	1.7	[32]
Germans	476	10.9	5.7	–	–	[33]
Italians	178	0.9	0.6	–	–	[34]
Finns	501	3.4	0.9	1.3	–	[35]

frequency of the FLG gene loss-of-function mutations (6.8%) compared with normal variability. In our study, the occurrence of R501X, S3247X and R2447X filaggrin gene mutations among AD patients was at the level of population variability for the cohort of conditionally healthy individuals. Thus, of the four studied *FLG* gene loss-of-function mutations, only 2282delACTG was associated with onset and development of AD in adult patients from the Russian Federation.

A comparison of the data on prevalence of the *FLG* gene loss-of-function mutations among adult AD patients in the Russian and European populations (Table 4) revealed slight fluctuations in the frequency of occurrence of the 2282delACTG mutation in different regions of the Russian Federation. The frequency variations, which, most likely, have evolutionary gene pool differences behind them, were twofold or less. The frequency of the 2282delACTG mutation in European adult AD patients varies more widely, and the value closest to that identified in our study was found in the respective reports from Finland. The data we obtained are comparable with the results of other studies investigating *FLG* gene loss-of-function mutations in Russian adult AD patients.

The integral frequency of the four *FLG* gene loss-of-function mutations (6.8%), as established in our study, was significantly lower than that registered in children with severe AD, which is 32.8% in the Russian population [21] and 42% in the comparable European patients [11]. A partial explanation of this phenomenon suggested by the researchers is that carriers of the loss-of-function mutations in the filaggrin gene have the diseases onset at an earlier age (the odds ratio in the group under 20 years is 8.9; in the group under 5 years — 7.8) [37]. The detailed classification of AD types based on the specifics of immune pathways of development of the disease (Th2/retinol, skin homing, Th1/Th2/Th17/IL1, and Th1/IL1 with the influence of eosinophils) can also explain the differences in the frequency of the *FLG* gene loss-of-function mutations in patients of different age groups [38, 39]. The severity of the disease and the pathogenesis of AD in adults are associated only with the 'skin homing' inflammation pathway, which is consistent with data on the prevalence of AD in 20% of children and 2–8% of adults [40].

CONCLUSIONS

The technique for detection of the four FLG gene loss-of-function mutations most common in the European populations was developed based on the SNaPshot (minisequencing) approach. Examination of patients with moderate to severe AD with this technique revealed following frequencies of mutations: 2282delACTG — 5.05%, R501X — 0.5%, R2447X — 1%. No S3247X mutation was detected in the sample. The level of the *FLG* gene loss-of-function mutations is comparable to that reported in other studies examining adult AD patients from Russia. The data obtained suggest that the filaggrin gene loss-of-function mutations most common in the European populations contribute to the development of AD insignificantly.

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