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Single-nucleotide polymorphism (rs11204981) in filaggrin gene and its functional significance for asthma among children with eczema

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The aim of this study was to determine whether SNP in filaggrin gene and expression of filaggrin mRNA in buccal epithelium are associated with childhood eczema and with the phenotype of childhood eczema combined with asthma. Genotyping for FLG (rs11204981) was performed in the following populations: patients with asthma (n = 99); ages 5-18 years (8 ± 2.1), and control group (n = 98); ages 5–18 years (12 ± 2.1) by using Real-time PCR. Level of mRNA expression was estimated by using reverse transcription and following real-time PCR. It was found out that 5.05 % of patients and 2.02 % of control group had minor allele (AA; P>0.05), 27.27 % and 36.36 % of patients and control group, respectively, had heterozygous allele (GA; P>0.05) and 67.68 % and 61.62 % had major allele (GG) (P>0.05). Variants with the AA-genotype of the FLG rs11204981 were found to be 2.5 times more frequently among patients than in control group. We also found out that the level of mRNA FLG expression in GG-genotype is 22.8 ± 11.67 (P>0.05 compared to AA-genotype), 92.95 ± 35.3 in GA genotype (P<0.05compared to GG-genotype) and 21.8 ± 13.4 in AA genotype (P>0.05 compared to GA-genotype). Thus, heterozygous variant has significantly higher expression of filaggrin in buccal epithelium. We suggest that SNP in FLG (rs11204981) may serve as an important predictive marker for the combined eczema plus asthma phenotype, and that the highest level of expression in heterozygous may have a protective role in developing allergy phenotype.
*Key words***:** *snp; filaggrin; asthma; paediatrics.*

INTRODUCTION

Eczema is a chronic inflammatory skin condition commonly associated with other atopic diseases, such as asthma. Genetic variants in epidermal proteins, such as filaggrin, are thought to play a critical role in the development of eczema and have been associated with the combination of eczema plus asthma [1].

Filaggrin is a major structural protein in the stratum corneum involved in the terminal differentiation of the epidermis and formation of the skin barrier [2]. The filaggrin gene (*FLG)* is located within the epidermal differentiation complex, a region on chromosome 1q21. Mutations in the filaggrin gene are the most significant known genetic factor for the development of atopic dermatitis [3]. Genetic variants (SNPs) in *FLG* also confer risk for the associated allergic diseases such as asthma [4-8]. Researches highlighted the importance of skin barrier function in the pathogenesis of atopic diseases and have motivated a surge in research characterizing the filaggrin-deficient skin barrier and its consequences. This reduction in barrier function may allow the development of inflammation due to the increased penetration of allergen through the skin allowing IgE sensitization. Studies of functional significance of genetic variants in FLG showed that this gene is likely to contribute to mechanisms by which a quantitative reduction in intracellular filaggrin (mRNA expression) levels results in the paracellular barrier defects [9].

We hypothesized that polymorphism rs11204981 in promoter region of *FLG* would be associated with childhood eczema and with the eczema plus asthma phenotype. To test this hypothesis, we examined differences in the frequency of this SNP between children without allergic pathology (control group) and children with eczema plus asthma and estimated that variants with the AA genotype of the *FLG* rs11204981 were at 2.5 times more frequent among patients than in control group. Functional analysis of this SNP showed that mRNA FLG expression in heterozygous is 4 times higher than GG-genotype ($P<0.05$) and 4.3 times higher than AA genotype ($P>0.05$).

METHODS

The control group included 98 children aged 5 to 18 years (12 ± 2.1) without allergic disease at the time of examination or history. Children in the control group had negative skin prick test and\or normal spirography results as well as negative data on the presence of allergic diseases in parents and close relatives.

The study included patients with asthma (n = 99); ages 5-18 years (8 ± 2/1). All children had symptoms of asthma, eczema or symptoms of eczema in anamnesis, allergic rhinitis, or at least one positive skin prick test result to a panel of 15 aeroallergens. Skin prick tests were carried out and interpreted by Global Allergy and Asthma European Network protocol, developed by using a common panel of inhalant allergens based on published practice guidelines, the European Academy of Allergy and Clinical Immunology (EAACI) Position Paper, the Nordic standards and the International Study of Asthma and Allergies in Childhood (ISAAC) phase II protocol. The research included children 5 to 18 years of age from subspecialty department at Kiev Children's Hospital and control subjects from the general population. All procedures performed in study were in accordance with the ethical standards of the O.Bogomolets National Medical University ethical committee and with the 1964 Helsinki declaration and its later amendments. Parents signed an informed consent prior to their inclusion in the study.

Outcome measures

The definition of eczema was adapted from a validated questionnaire (ISAAC) and included a parental report of the child's scratching *and* redness, "raised bumps" or dry skin/scaling during the childhood. Cases with eczema included children with a physician's diagnosis or a parental report of eczema. Physical examination findings are considered to be consistent with eczema including erythema, papulation, excoriations, and/or lichenification. Testing for asthma were performed; the definition included a parental report of persistent wheezing (≥2 wheezing episodes that is not associated with a cold or upper respiratory tract infection), spirometry (a reduced FEV_1 and FEV_1/FVC ratio, positive test with β_2-agonists), increased IgE level, positive skin prick tests with aeroallergens (Table 4).

Selection of SNP

An SNP rs11204981 in *FLG*, which was located in promoter region, was selected for genotyping, as it hypothetically can affect expression of *FLG* mRNA and is reported to be common in European populations.

DNA and RNA extraction

Buccal epithelium was taken by using buccal brushes with the following freezing of samples and their storage at -20ºC. DNA for genotyping was extracted from the samples by using Isogene kits (Russian Federation) according to manufacturer's protocol. RNA was extracted from buccal epithelium samples by using phenol-chloroform extraction. The concentration of total DNA and RNA was determined by using a NanoDrop spectrophotometer ND1000 (NanoDrop Technologies Inc., USA).

qPCR Genotyping

Amplification reactions were performed by using a 7500 Fast Real-time PCR System ("Ap-

plied Biosystems", USA) in a final reaction volume of 20 µl, which contained 2X TaqMan Universal Master Mix ("Applied Biosystems", USA), assayC_1792560_10 and the template cDNA. The thermal cycling conditions involved a denaturation step at 95°C for 20 s, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. Analysis of the data was carried out with 7500 Fast Real-Time PCR Software.

Reverse transcription and real-time PCR

RNA was reverse transcribed with random hexamer primers by using the RevertAid First Strand cDNA Synthesis Kit ("Thermo Scientific", USA). Single-strand obtained from cDNA was used for real-time PCR. Gene expression of *FLG* was determined by using the TaqMan gene expression assay (Hs 00856927_g1, Applied Biosystems, USA). The pairs of forward and reverse primers for the genes indicated above and the TaqMan probes for the target mRNAs were designed based on the human mRNA sequence by "Applied Biosystems". Gene expression in each probe was normalized to β-actin, as a housekeeping gene, by using TaqMan β-actin control reagent (FAM Probe). The thermal cycles of PCR amplification were the following: initial denaturation step at 95°C for 20 s, and treatment at 95°C for 3 s and 60°C for 30 s (45 cycles) with the use of 7500 Fast Real-Time PCR ("Applied Biosystems"). Analysis of the data was carried out with 7500 Fast Real-Time PCR Software.

Statistical analysis

χ^2 test was performed to investigate if there was any difference in the frequency of the genotype and allele between the asthma patient group and the normal control group. A P-value of less than 0.05 ($P<0.05$) was considered statistically significant. Frequency distribution of genotypes was analyzed by using Pearson chi-square test with Excel 2010 software. SNPAnalyzer (web-based software) was used to examine Hardy-Weinberg equilibrium.

RESULTS

100% of children have atopic dermatitis in history, 35.35 % of children have manifestations of atopic dermatitis in the form of rashes, dry skin, peeling, pruritus, lichenification during last year. 80.81% had mild severity on a scale SCORAD, 20.20 % – average severity. Other concomitant allergic pathology was determined in 46 % of children (allergic rhinitis, food allergy). 72.73 % of children were in a state of exacerbation of asthma, manifested in wheezing and cough (of which 60.61 % had mild exacerbation severity, 39.39 % had moderate severity of exacerbation), 28.28 % of children had remission. 60.61 % of patients had decreased FEV_1 (<80%), 34.34 % had positive test with β_2-agonist.

Characteristics of the patients with atopic dermatitis and asthma:

	Value
Median age (years) (n=99)	8 ± 2.1
Sex ratio (% male) (n=99)	57.6%
Mean SCORAD (CI) (n=51)	20 (16 – 24)
Median total IgE, kU•l–1 (IQ) (n=24)	821.4 (42 – 8000)
AD prevalence (n=99)	35%
FEV1, % (n=76)	76.4 (28.64 – 117.8)
FEV1/FVC ratio (n=76)	106.74 (84.4 – 118.39)
FEV1 improvement after salbutamol (n=76)	12.5 (0.4 – 87.2)

Genotyping

We found that SNP was in Hardy-Weinberg equilibrium. Our main results are as follows: 5.05 % of patients and 2.02 % of control group had minor allele (AA; $P> 0.05$ by χ^2–test, OR – 2.28 (95%CI 0.42–1.2), 27.27 % and 36.36 % of patients and control group, respectively, had heterozygous allele (GA; $P> 0.05$ by

χ^2–test, OR – 1,69 (95% CI 0.92-3.1) and 67.68 % and 61.62 % had major allele (GG; P>0.05 by χ^2–test). Variants with the AA-genotype of the *FLG* rs11204981 were found to be 2.4 times more frequent among patients than in control group (Tables 1-3, Fig 1, 2).

Table 1. Odds ratio for co-dominant inheritance model in children with asthma. Odds ratios with 95% confidence intervals.

Genotype	Genotype (n)		Odds Ratio and 95% CI
	Control	Patients	
GG	61	67	1 (ref)
GA	36	27	1.69 (0.92-3.1)
AA	2	5	2.28 (0.42-12.2)

Table 2. Odds ratio for dominant inheritance model in children with asthma. Odds ratios with 95% confidence intervals.

Genotype	Genotype (n)		Odds Ratio and 95% CI
	Control	Patients	
GG	61	67	1
GA/AA	38	32	0.77 (0.43-1.38)

Table 3. Odds ratio for recessive inheritance model in children with asthma. Odds ratios with 95% confidence intervals.

Genotype	Genotype (n)		Odds Ratio and 95% CI
	Patients	Control	
GG/GA	97	94	1
AA	2	5	2.58(0.49-13.62)

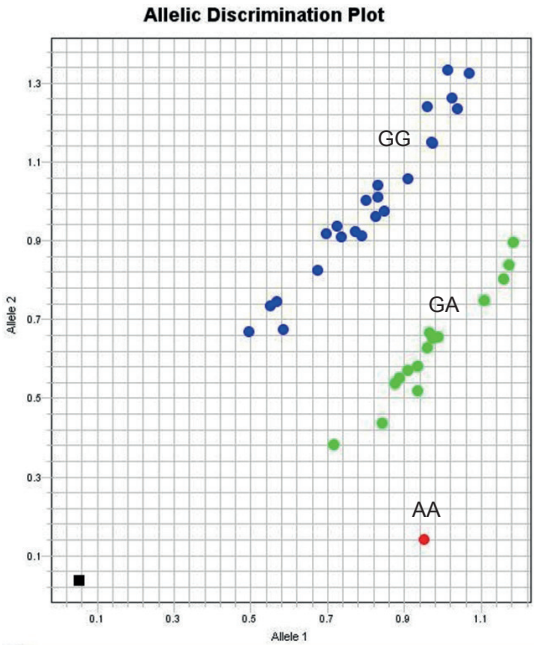

Fig. 1 Allelic discrimination plot for real time PCR results detecting the homozygous genotype GG, heterozygous GA and homozygous AA. Minor allele (AA) was present at 5.05 % of patients, 27.27 % had heterozygous allele (GA) and 67.68 % had major allele (GG)

FIG expression

We also found that the level of mRNA FLG expression in GG-genotype is 22.8 ± 11.67 (P>0.05 compared to AA-genotype), 92.95 ± 35.3 in GA-genotype (P<0.05 compared to GG-genotype) and 21.8 ± 13.4 in AA genotype (P>0.05 compared to GA-genotype). Thus, heterozygous variant has significantly higher expression of filaggrin in buccal epithelium (Fig 2).

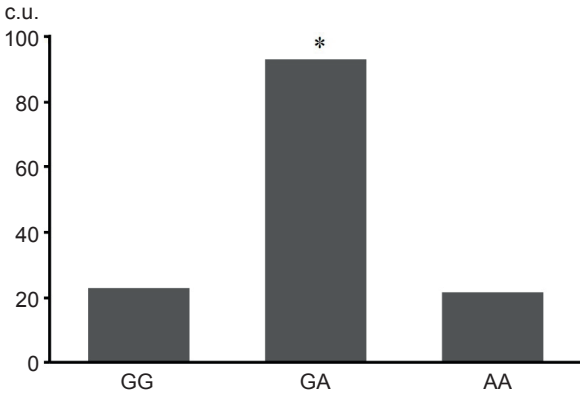

Fig.2 Messenger ribonucleic acid expression depending on FLG genotype

DISCUSSION

Evidence from the literature has established that epidermal barrier function plays a critical role in eczema and possibly in the phenotype of eczema plus asthma. The combination of eczema plus asthma may represent a distinct endophenotype with unique pathogenic and prognostic implications. Identification of risk factors that may modify the risk of asthma among children with eczema is important. In addition, improved understanding of the relationship between eczema and asthma may provide mechanistic insights into the pathogenesis of asthma. We explored the relationship of childhood eczema plus asthma with epithelial protein encoded on 1q21 as well as with data from RNA expression studies and allele frequency differences in asthmatic populations. We demonstrated a possible connection between an SNP in *FLG* and eczema plus asthma among children. The AA genotype of the rs11204981 SNP conferred an approximately 2.5 times increased risk for eczema plus asthma phenotype. Findings for RNA filaggrin expression in buccal epithelium appear to be related to *FLG* polymorphism. Thus FLG polymorphism may affect filaggrin expression in the skin.

In this article, we determined the frequency of the rs11204981 SNP and showed that minor allele occurs 2.4 times more frequently among patients, although there was no statistical significance. Association of genotype with clinical parameters also was not established previously. Some researchers demonstrated association of an increased risk of developing atopic dermatitis with other FLG polymorphisms. In particular, positive associations of rs2184951 and rs12730241 [6] with asthma and allergic diseases were determined. Polymorphism rs11204981 has been widely studied in the European population and therefore was chosen by us for this research.

However, in the study of the expression level of the filaggrin mRNA we were able to show that this polymorphism is functional and affects the expression of filaggrin. mRNA FLG expression in heterozygote is 4 times higher than common homozygous genotype ($P<0.05$) and 4.3 times higher than homozygous risk genotype ($P>0.05$).

CONCLUSIONS

Our research has demonstrated functional significance of heterozygote genotype of *FLG* polymorphism, thus suggesting that this polymorphism may have a predictive value in the development of asthma and eczema affecting the expression of that gene. Thereby, polymorphisms in *FLG* may independently serve as useful markers to predict the development of asthma among children with eczema.

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ФУНКЦІОНАЛЬНЕ ЗНАЧЕННЯ ОДНОНУКЛЕОТИДНОГО ПОЛІМОРФІЗМУ (RS11204981) В ГЕНІ ФІЛАГРІНУ (FLG) ДЛЯ ЛІКУВАННЯ БРОНХІАЛЬНОЇ АСТМИ У ДІТЕЙ З АТОПІЧНИМ ДЕРМАТИТОМ.

Метою нашого дослідження було визначити зв'язок однонуклеотидного поліморфізму в гені філагріну і експресії його мРНК в букальному епітелії та фенотипом бронхіальної астми з супутнім атопічним дерматитом у дітей. Генотипування філагріну (rs11204981) проводили в групі хворих з бронхіальною астмою (n=99) віком 5-18 років (8 ± 2,1 років) і контрольній групі (n=98) віком 5–18 років (12 ± 2,1 років)за допомогою полімеразної ланцюгової реакції (ПЛР) у реальному часі. Рівень експресії мРНК оцінювали з використанням зворотної транскрипції та наступної ПЛР у реальному часі. Ми виявили, що у 5,05 % пацієнтів і у 2,02 % здорових дітей був мінорний алельний варіант (АА), 27,27 і 36,36 % відповідно мали гетерозиготний алель (GA), 67,68 і 61,62 % – мажорний алель (GG). Варіанти з генотипом АА з rs11204981 FLG виявилися в 2,4 раза частіше у пацієнтів, ніж у контрольній групі. Нами було виявлено, що рівень експресії мРНК філагріну у пацієнтів з GG-генотипом становить 22,80±11,67 ($P>0,05$ порівняно з генотипом АА), 92,95±35,30 в GA-генотипі ($P<0,05$ порівняно з GG-генотипом) і 21,80±13,40 у генотипі АА ($P>0,05$ порівняно з GA-генотипом). Таким чином, гетерозиготний варіант має значно вищу експресію філагріну в епітелії слизової оболонки рота. Ми вважаємо, що поліморфізм у гені *FLG* (rs11204981) може бути важливим прогностичним маркером для фенотипу бронхіальної астми та атопічного дерматиту, і що високий рівень експресії в гетерозиготному стані може свідчити про протекторну роль цього генотипу у розвитку алергії.

Ключові слова: однонуклеотидний поліморфізм; філагрін; астма; педіатрія.

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ФУНКЦИОНАЛЬНОЕ ЗНАЧЕНИЕ ОДНОНУКЛЕОТИДНОГО ПОЛИМОРФИЗМА (RS11204981) В ГЕНЕ ФИЛАГРИН (FLG) ДЛЯ ЛЕЧЕНИЯ БРОНХИАЛЬНОЙ АСТМЫ У ДЕТЕЙ С АТОПИЧЕСКИМ ДЕРМАТИТОМ

Целью нашего исследования было определить связь однонуклеотидного полиморфизма в гене филагрина и экспрессии его мРНК в буккальном эпителии и фенотипа бронхиальной астмы с сопутствующим атопическим дерматитом у детей. Генотипирование филагрина (rs11204981) проводили в группе больных с бронхиальной астмой (n = 99) в возрасте 5-18 лет (8±2,1 лет) и контрольной группе (n = 98) в возрасте 5-18 лет (12±2,1 лет) с помощью полимеразной цепной реакции (ПЦР) в реальном времени. Уровень экспрессии мРНК оценивали с использованием обратной транскрипции и последующей ПЦР в реальном времени. Мы обнаружили, что у 5,05% пациентов и у 2,02% здоровых детей был минорный аллельный вариант (AA), 27,27 и 36,36% соответственно имели гетерозиготный аллель (GA), 67,68 и 61,62% – мажорный аллель (GG). Варианты с генотипом AA rs11204981 гена *FLG* оказались в 2,4 раза чаще у пациентов, чем в контрольной группе. Нами было выявлено, что уровень экспрессии мРНК филагрина у пациентов с GG-генотипом составляет 22,80±11,67 ($P>0,05$ по сравнению с генотипом AA), 92,95±35,30 в GA-генотипе ($P<0,05$ по сравнению с GG-генотипом) и 21,80±13,40 в генотипе AA ($P>0,05$ по сравнению с GA-генотипом). Таким образом, гетерозиготный вариант имеет значительно более высокую экспрессию филагрина в эпителии слизистой оболочки рта. Мы считаем, что полиморфизм в гене *FLG* (rs11204981) может быть важным прогностическим маркером для фенотипа бронхиальной астмы и атопического дерматита, и высокий уровень экспрессии в гетерозиготном состоянии может свидетельствовать о протекторной роли этого генотипа в развитии аллергии.

Ключевые слова: однонуклеотидный полиморфизм; филагрин; астма, педиатрия.

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