การวิเคราะห์การกลายพันธุ์ของยีน *Filaggrin*, *SLC25A46* และ *TMEM232*

ในผู้ป่วยไทยที่เป็น Atopic dermatitis

นางสาวปิยะไม จันทร์เกษ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Mutation analysis of *Filaggrin*, *SLC25A46* and *TMEM232* in Thai patients with atopic dermatitis

Miss Piyamai Changate

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Thesis Title	MUTATION ANALYSIS OF *FLG, SLC25A46* AND *TMEM232* IN THAI PATIENTS WITH ATOPIC DERMATITIS
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Field of Study	Medical Science
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ปิยะไม จันทร์เกษ : การวิเคราะห์การกลายพันธุ์ของยีน *Filaggrin, SLC25A46* และ *TMEM232* ในผู้ป่วยไทยที่เป็นโรค atopic dermatitis (Mutation analysis of *Filaggrin, SLC25A46* and *TMEM232* in Thai patients with atopic dermatitis) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.พญ.ดร.กัญญา ศุภปีติพร , อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ.นพ. วรศักดิ์ โชติเลอศักดิ์, 72 หน้า.

Atopic dermatitis เป็นโรคผิวหนังอักเสบเรื้อรังชนิดหนึ่งซึ่งส่งผลกระทบต่อคุณภาพชีวิตของผู้ป่วย สาเหตุของโรคเกิดจากหลายปัจจัยร่วมกัน ได้แก่ พันธุกรรม และสิ่งแวดล้อม ปัจจุบันมีรายงานว่าการกลายพันธุ์แบบสูญเสียการทำงานของยีน filaggrin (*FLG*) เป็นปัจจัยทางพันธุกรรมที่สำคัญต่อการเกิดโรค atopic dermatitis ในชาวยุโรปและเอเชีย อย่างไรก็ตามยัง ไม่เคยมีการศึกษาความสัมพันธ์ของการกลายพันธุ์ในยีน *FLG* กับผู้ป่วย ไทย atopic dermatitis มาก่อน นอกจากนี้ การศึกษา genome-wide association ในผู้ป่วย atopic dermatitis เชื้อชาติจีน พบตำแหน่งใหม่ที่เกี่ยวข้องกับโรค ซึ่งประกอบด้วยยีน *SLC25A46* และ *TMEM232* ยังไม่มีการศึกษาการกลายพันธุ์ในยีนดังกล่าวมาก่อน ดังนั้น ทีม ผู้วิจัยจึงได้ทำการศึกษาการกลายพันธุ์ของยีน *FLG, SLC25A46* และ *TMEM232* ในผู้ป่วยไทยที่เป็นโรค atopic dermatitis จำนวน 49 ราย โดยวิธี PCR-sequencing เพียงอย่างเดียว ซึ่งถูกพัฒนาขึ้นเพื่อช่วยลดต้นทุน ประหยัดเวลาในการศึกษา และง่ายต่อการตรวจหาการกลายพันธุ์ ผลการศึกษาพบการกลายพันธุ์ในยีน *FLG* จำนวน 4 ตำแหน่ง ได้แก่ p.G526X, p.R1555SfsX1706, p.S1906X, และ p.R3982X ในผู้ป่วยจำนวน 4 ราย จากผู้ป่วยทั้งหมด 48 ราย คิดเป็นร้อยละ 8.3 และการกลายพันธุ์ทั้ง 4 แบบนี้ยังไม่เคยมีการรายงานมาก่อน อีกทั้งยังมีหลักฐานหลายประการบ่งชี้ว่าการกลายพันธุ์ดังกล่าวมีบทบาทต่อการเกิดโรค อย่างไรก็ตาม ไม่พบการกลายพันธุ์ที่คาดว่าจะเป็นสาเหตุของโรคในยีน *SLC25A46* และ *TMEM232* โดยสรุป การศึกษานี้พบการกลายพันธุ์ใหม่ชนิดสูญเสียการทำงานในยีน *FLG* 4 ตำแหน่ง ซึ่งเป็นการเพิ่ม mutational spectrum ในยีนนี้ และได้แสดงถึงความสำคัญของการกลายพันธุ์ในยีน *FLG* ต่อการเกิดโรค atopic dermatitis ในประชากรไทย

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2555 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

##547 41396 30: MAJOR MEDICAL SCIENCE
KEYWORDS: FILAGGRIN/ ATOPIC DERMATITIS/ NOVEL MUTATION

PIYAMAI CHANKATE: MUTAYION ANALYSIS OF *FILAGGRIN*, *SLC25A46*, AND *TMEM232* IN THAI PATIENTS WITH ATOPIC DERMATITIS. THESIS ADVISOR: ASSOC. PROF. KANYA SUPHAPEETIPORN, M.D., Ph.D., THESIS CO-ADVISOR: PROF. VORASUK SHOTELERSUK, M.D., 72 pp.

Atopic dermatitis is a chronic inflammatory skin disease that affects patients' quality of life. It has complex etiology with genetic and environmental factors. Recently, loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as its major predisposing factor in some Caucasian and Asian populations. FLG plays an essential role in the terminal differentiation of the epidermis and formation of skin barrier. However, the association between *FLG* mutations and atopic dermatitis in the Thai population remains unknown. In addition, Genome-wide association study in the Han Chinese population revealed a new susceptibility locus for atopic dermatitis comprising *SLC25A46* and *TMEM232*. Here we investigated the *FLG, SLC25A46,* and *TMEM232* mutations by PCR-sequencing of the entire coding regions of *FLG* in 48 unrelated Thai individuals with atopic dermatitis. Our modified PCR method helped reduce operating costs and time. Four *FLG* mutations, p.G526X, p.R1555SfsX1706, p.S1906X, and p.R3982X were identified in four unrelated patients (8.3%). All four mutations have never been previously reported. Several lines of evidence suggest their pathogenicity. We found no pathogenic mutations in *SLC25A46* and *TMEM232*. In summary, for the first time, we reported four *FLG* novel mutations in Thai atopic dermatitis patients expanding mutational spectrum of *FLG* and suggested the important role of *FLG* in Thai patients with atopic dermatitis.

Field of Study: Medical Science Student's Signature……..…
Academic Year: 2012 Advisor's Signature…….……..…
Co- advisor's Signature……

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LIST OF ABBREVIATIONS

GWAS	=	Genome-wide association study
FLG	=	Filaggrin
SLC25A46	=	Solute carrier family 25, member 46
TMEM232	=	Transmembrane protein 232
IgE	=	Immunoglobilin E
PCR	=	Polymerase chain reaction
SNPs	=	Single nucleotide polymorphisms

CHAPTER I

INTRODUCTION

Background and rational

Atopic dermatitis is a chronic inflammatory skin disease characterized by xerosis, pruritus, lichenification and eczematous lesions that occur in different areas of the body and vary with age.[1] Most patients have elevated total serum IgE levels and specific IgE to environmental and food allergens. They have also been found to be associated with other atopic disorders, such as allergic rhinitis, and asthma. Some of the patients however show normal IgE levels and the absence of specific IgE. [2] The histological features of atopic dermatitis reveal infiltration of lymphocytes, macrophages, dendritic cells and a few eosinophils in dermis.[3] Atopic dermatitis is one of the most common diseases in children, affecting 10.1% of Thai children aged 0–5 years in 2010.[4] Diagnosis of atopic dermatitis requires at least 3 major criteria plus 3 or more minor criteria established by Hanifin and Rajka Diagnostic criteria for atopic dermatitis.[5] Atopic dermatitis is a chronic disease that affects patients' quality of life in the long term. There is no cure for atopic dermatitis, but short-term treatment to relieve the symptoms is available. Therefore, understanding the biology of the disease can facilitate the development of effective treatment in the future.

Atopic dermatitis results from a complex interaction between genetic and environmental factors. However, the pathogenesis of this disease remains unclear. Several lines of evidence suggest that genetic factors contribute to atopic dermatitis. Previous studies demostrated that children whose parents had atopic dermatitis showed a risk of developing atopic dermatitis. There was a strong association between atopic dermatitis in parents and atopic dermatitis in offspring.[6] In addition, twin studies have revealed the concordance rate in monozygotic twins (86%) is higher than that in dizygotic twins (21%).[7] However, the concordance rate in monozygotic twins is far from 100%. These findings have suggested that environmental factors are operating in genetically susceptible persons. Several approaches for example genome-wide linkage analysis, genome-wide association study (GWAS) and candidate gene analysis have been performed to identify genetic factors contributing to atopic

dermatitis. Several susceptibility loci for atopic dermatitis have been identified on chromosomes 1q21, 3q21, 3p24, 4q22.1, 5q31-q33, 11q13.5, 13q12-q14, 17q25.3 and 20p.[8-13] There are 46 genes having at least one positive association with atopic dermatitis.[14] These genes can be classified into two major groups on the basis of pathophysiology of atopic dermatitis. The first group includes genes involved in the immune response. Polymorphisms in these genes are thought to promote the switch to Th2 immune response, increased IgE production and increased vascular permeability and accumulation of inflammatory cells in chronic atopic dermatitis skin lesions.[15] However, several studies have revealed negative associations between genes involved in the immune response with atopic dermatitis.[16] The second group includes genes encoding proteins for skin barrier function, such as genes encoding epidermal structural proteins, epidermal proteases and protease inhibitors.[17] Defects of skin barrier function can promote increased trans-epidermal water loss and allow penetration of allergens and microbes from environment through the skin resulting in inflammation, and allergen sensitization in atopic dermatitis patients. It has been proposed that excess of immune responses and impairment of skin barrier drive skin inflammation in atopic dermatitis.

Recently, loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as a major predisposing factor of atopic dermatitis in some Caucasian and Asian populations.[18-22] Approximately 20 studies in Europe and Asia reported a strong and significance association between *FLG* and atopic dermatitis.(14) However, the association between *FLG* mutations and atopic dermatitis in the Thai population remains unknown. Filaggrin plays an essential role in the terminal differentiation of the epidermis and formation of skin barrier.[23] An animal model study in flaky tail mice harboring the 1-bp deletion mutation in murine *Flg* provided evidence suggesting that filaggrin deficiency led to penetration of antigen through the impaired skin barrier and development of skin inflammation with atopic disease.[24] Therefore, *FLG* is one of the most important candidate genes for atopic dermatitis.

In 2011, there was a GWAS report in the Han Chinese population. They identified a new susceptibility locus of atopic dermatitis at 5q22.1 (*TMEM232* and *SLC25A46*, rs7701890, *P* combined = 3.15×10^{-9}, odds ratio = 1.24). [25]

TMEM232 encodes a transmembrane protein 232 belonging to the functional class of transmembrane proteins, and *SLC25A46* belongs to the solute carrier 25 (SLC25) family which encodes mitochondrial carrier proteins, and could shuttle metabolites across the inner mitochondrial membrane.[25] The exact functions of both genes remain unclear.

Although several genome-wide associations for atopic dermatitis have been conducted, the underlying genes remained elusive. The aim of our study was to investigate the *FLG, SLC25A46, and TMEM232* mutations by PCR-sequencing of the entire coding regions of *FLG*, *SLC25A46, and TMEM232* in 48 unrelated Thai individuals with atopic dermatitis.

Research questions

1. What percentages of Thai patients with atopic dermatitis are caused by mutations in the *FLG* gene*?*
2. Do mutations in *SLC25A46* and *TMEM232* underlie atopic dermatitis?

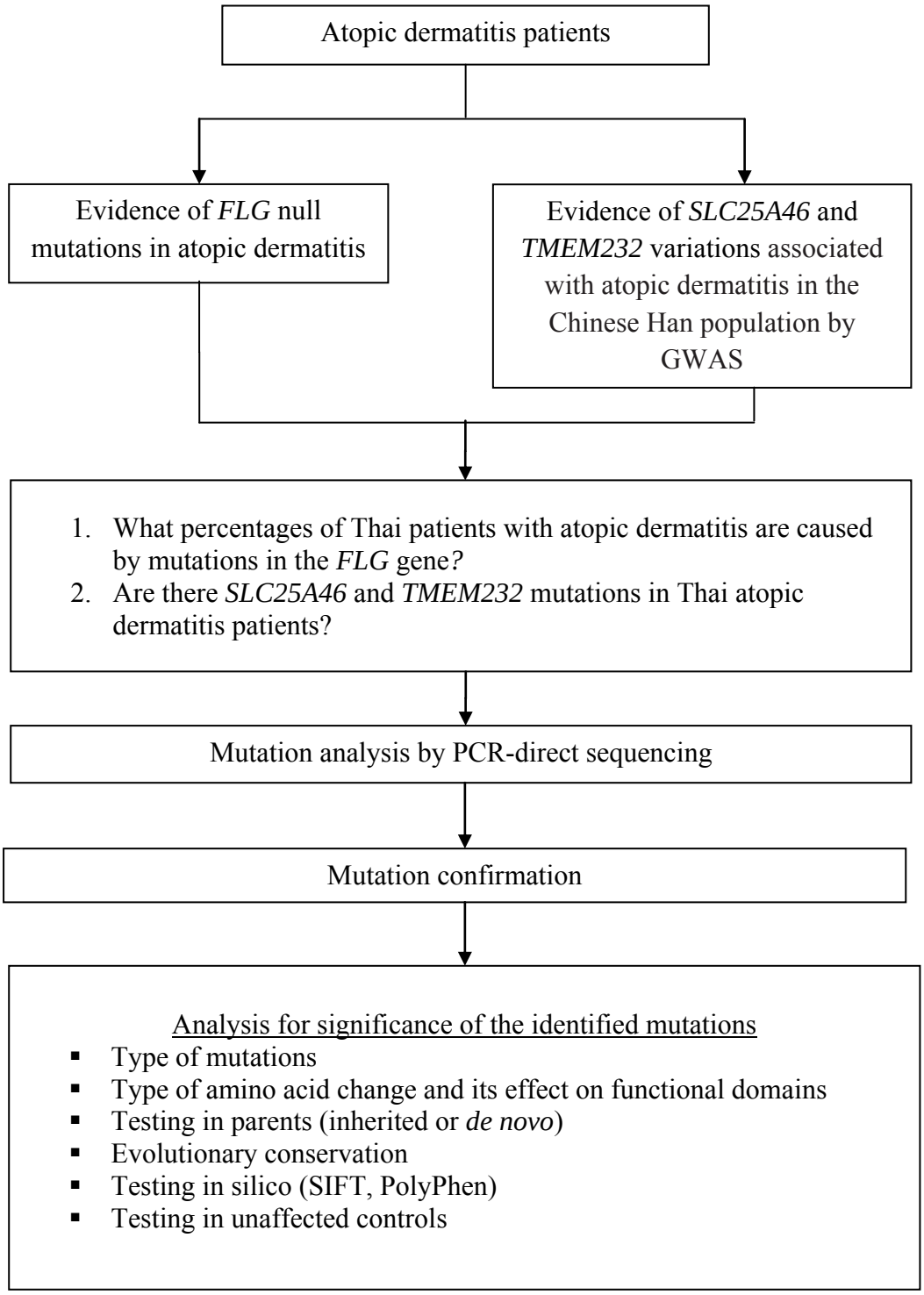
Objective

1. To investigated the percentages of *FLG* mutations in Thai patients with atopic dermatitis.
2. To investigated the *SLC25A46, and TMEM232* mutations in Thai patients with atopic dermatitis.

Expected benefits

This is the first study to investigate the mutations of *FLG*, *SLC25A46*, and *TMEM232* in Thai patients with atopic dermatitis. The study could provide further insight into the biology of atopic dermatitis which is a multifactorial disorder and its etiology remains unclear. Development in genetic tests could lead to significant benefits in improving patient management including genetic counseling, and more accurate diagnosis. Furthermore, the identification of genetic defects in patients with atopic dermatitis may facilitate the development of novel treatments to repair or replace the defective skin barrier.

Conceptual framework



Research Design

Descriptive and *in vitro* studies

Ethical Consideration

This work was approved by the Ethics Committee of the Institutional Review Board of Faculty of Medicine, Chulalongkorn University. Written informed consent was obtained from all patients or their parents who participated in the study.

Research Methodology

1. Sample collection

1.1 Cases are 48 Thai patients with atopic dermatitis diagnosed by pediatric dermatology at King Chulalongkorn Memorial Hospital. Initial diagnosis is based on the patients' clinical features.

1.2 Controls are 50 unrelated ethnically-matched healthy blood donors who are unaffected with skin disorders, atopic disease and have no family history of atopic dermatitis and atopic disease.

2. Study process

2.1 Blood collection

2.2 Mutation analysis

DNA extraction

DNA amplification

Agarose gel electrophoresis

DNA sequencing

2.3 Mutation confirmation and screening

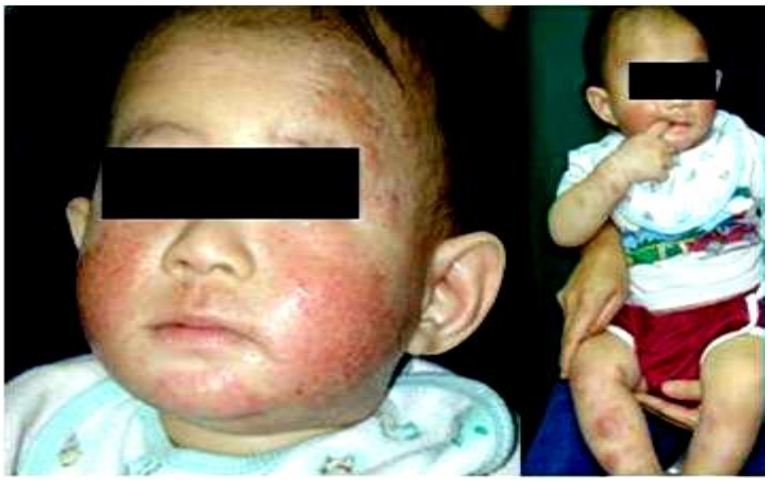
3. Data collection and analysis for significance of the identified mutations

CHAPTER II
LITERATURE REVIEW

Clinical manifestation of atopic dermatitis

Atopic dermatitis is a chronic inflammatory skin disease characterized by xerosis, pruritus, lichenification and eczematous lesions that occur in different areas of the body and vary with age [1] (figure 1). It can be divided in to two types of atopic dermatitis: extrinsic or allergic atopic dermatitis shows elevated total serum IgE levels and the presence of specific IgE for environmental, food allergens and found to be associated with other atopic disorders, such as allergic rhinitis, and asthma whereas intrinsic or non-allergic atopic dermatitis shows normal IgE levels and the absence of specific IgE [2]. The histological features of atopic dermatitis show infiltration of lymphocytes, macrophages, dendritic cells and a few eosinophils in dermis [3]. Atopic dermatitis is one of the most common diseases in children, affecting 10.1% of Thai children aged 0–5 years in 2010 [4]. Diagnosis of atopic dermatitis requires at least 3 major criteria plus 3 or more minor criteria established by Hanifin and Rajka Diagnostic criteria for atopic dermatitis (table 1) [5].

A



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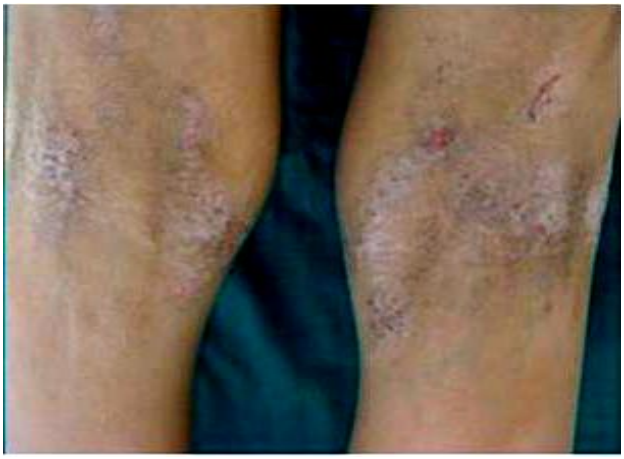


Figure 1. Clinical manifestations of atopic dermatitis. (A) Eczematous lesions appear on scalp and cheeks in a child. (B) Lichenified plaques affect the flexure of arms and legs in an adult.

Table 1. Hanifin and Rajka's Diagnostic criteria for atopic dermatitis [5]

Major criteria	Minor criteria
1. Pruritus 2. Typical morphology and distribution Adults: Flexural lichenification Infants and children: Facial and extensor involvement 3. Chronic or chronically-relapsing dermatitis 4. Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)	1. Xerosis 2. Ichthyosis, palmar hyperlinearity, or keratosis pilaris 3. Immediate (type 1) skin-test reactivity 4. Raised serum IgE 5. Early age of onset 6. Tendency toward cutaneous infections (especially *S aureus* and herpes simplex) or impaired cell-mediated immunity 7. Tendency toward non-specific hand or foot dermatitis 8. Nipple eczema 9. Cheilitis 10. Recurrent conjunctivitis 11. Dennie-Morgan infraorbital fold 12. Keratoconus 13. Anterior subcapsular cataracts 14. Orbital darkening 15. Facial pallor or facial erythema 16. Pityriasis alba 17. Anterior neck folds 18. Itch when sweating 19. Intolerance to wool and solvents 20. Perifollicular accentuation 21. Food intolerance 22. Course influenced by environmental or emotional factors 23. White dermographism

Etiology of atopic dermatitis

The etiology of atopic dermatitis remains unclear. However, there are two major factors contributing to atopic dermatitis pathogenesis comprising of genetic factors and environmental factors. Several lines of evidence show that genetic factor contributes to atopic dermatitis. Children whose parents have atopic dermatitis show a high risk of developing atopic dermatitis [6]. In addition, twin study revealed the concordance rate in monozygotic twins (86%) was higher than dizygotic twins (21%) [7]. However, the concordance in monozygotic twins is far from 100%. It is suggested that environmental factors are operating in genetically susceptible persons. Several susceptibility loci for atopic dermatitis have been identified on chromosome 1q21, 3q21, 3p24, 4q22.1, 5q31-q33, 11q13.5, 13q12-q14, 17q25.3 and 20p.[8-13] There are 46 genes associated with atopic dermatitis.[14]

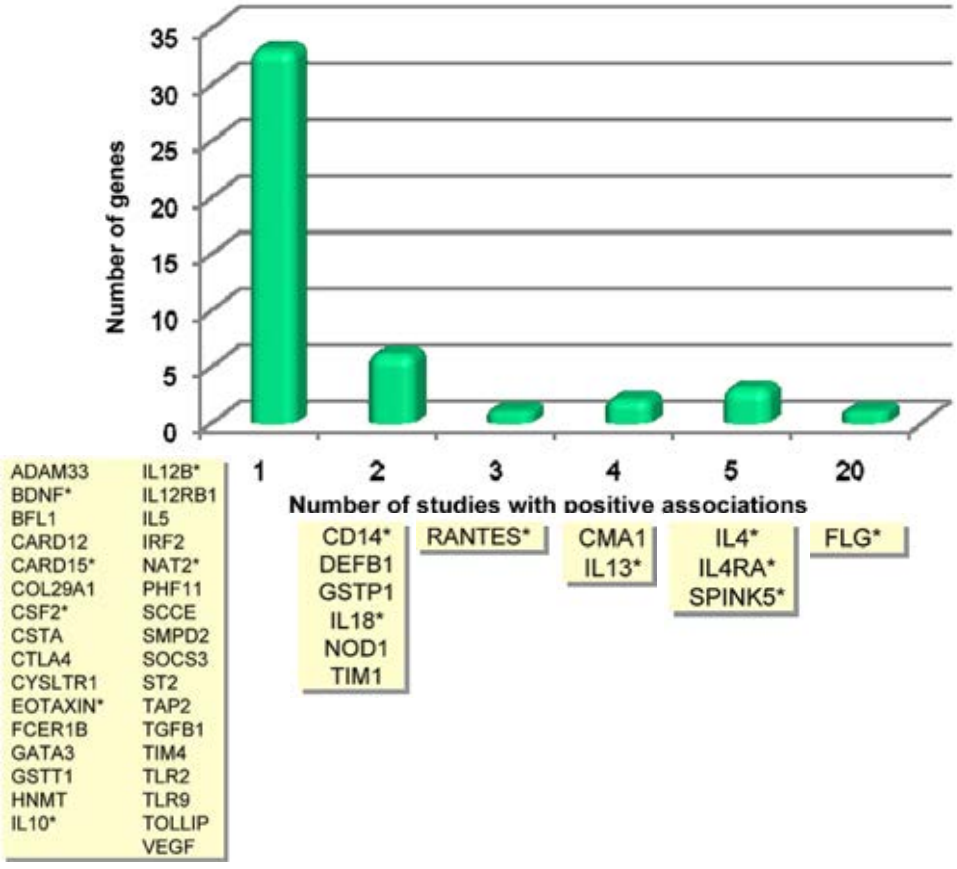


Figure 2. Genes associated with atopic dermatitis. The X-axis indicates the number of studies that showed positive association correlated with gene in each box. The Y-axis indicates the number of genes (corresponding to the boxes) for each positive association. [14]

These genes can be classified into two major groups on the basis of pathophysiology of atopic dermatitis.

First group composes of genes involving in the immune response (Table 2). However, many studies in different ethnic group revealed negative associations between genes involving in the immune response with atopic dermatitis [16].

Second group includes genes encoding proteins for skin barrier function: genes encoding epidermal structural proteins, epidermal proteases and protease inhibitors (Table 2). Defect of skin barrier function promotes increased trans-epidermal water loss and allows penetration of allergens and microbes from environment through the skin resulting in inflammation, and allergen sensitization in atopic dermatitis patients. For example, *CLDN1* encodes Claudin-1 tight junction protein which reduces expression in atopic dermatitis patients. Its haplotype-tagging SNPs revealed association with atopic dermatitis in North American populations [26]. Another interesting gene is *COL29A1* which encodes an epidermal collagen. Lack of COL29A1 expression in the outer epidermis of atopic dermatitis patients points to a role of collagen XXIX in epidermal integrity and function [27]. Recently, loss-of-function mutations in the filaggrin gene (*FLG*) have been identified as its major predisposing factor in some Caucasian and Asian populations [18-22]. Approximately 20 studies from Europe and Asia reported a strong and significant association between *FLG* and atopic dermatitis [14]. *FLG* is one of the most candidate genes for atopic dermatitis because *FLG* not only shows a strong association with atopic dermatitis but also plays an essential role in the terminal differentiation of the epidermis and formation of skin barrier.

Table 2. Candidate genes for Atopic dermatitis that have been associated with atopic dermatitis in at least one study

Susceptibility gene	Function	Variant	Phenotype	Population	Reference
Interleukin-4 (*IL4*)	Promotes the switch to Th_2 immune response and IgE production	-589 C/T	Atopic dermatitis	Japanese	[28]
			Extrinsic atopic dermatitis	German	[29]
Interleukin-4 receptor (*IL4R*)	Promotes the switch to Th_2 immune response and IgE production	Gln551Arg	Severe atopic dermatitis	American	[30]
			Adult atopic dermatitis	Japanese	[31]
			Atopic dermatitis	British	[32]
		−3223 C/T	Extrinsic atopic dermatitis	German	[29]
			Atopic dermatitis	Japanese	[33]
IL13 Interleukin-13	Promotes B-cell switch to IgE production	Arg130Gln	Atopic dermatitis	Canadian	[34]
			Atopic dermatitis	Japanese	[35]
			Atopic dermatitis	German	[36]
		-1112 C/T	Atopic dermatitis	Dutch	[37]

Susceptibility gene	Function	Variant	Phenotype	Population	Reference
IL12B Interleukin-12B	Play an important role in inducing Th1 type cytokine profiles	1188 A/C	Atopic dermatitis, Psoriasis	Japanese	[38]
IRF2 Interferon regulatory factor 2	Modulate cellular responses to interferons and viral infection as well as regulate cell growth and transformation. *Irf2* knockout mice show Th1 cell development defect and development of inflammatory skin disease	−467 G/A	Atopic dermatitis	Japanese	[39]
CSF2 Colony-stimulating factor 2	Promotes the maturation and migration of bone marrow precursors to form epidermal Langerhans cells and subsequently the migration of these Langerhans cells to draining lymph nodes to activate T lymphocytes.	−677 A/C 3606 T/C, 3928 C/T	Atopic dermatitis Atopic dermatitis at 12 and 24 months	British Canadian	[40] [41]

Susceptibility gene	Function	Variant	Phenotype	Population	Reference
CD14 Monocyte differentiation antigen CD14	Encoding part of the endotoxin receptor	−159 C/T	Atopic dermatitis (interaction with dog ownership)	American	[42]
CMA1 Mast cell chymase	Promotes increased microvascular permeability and accumulation of inflammatory cells	BstXI	Atopic dermatitis	Japanese	[43]
RANTES Regulated on activation, normally T cell expressed and secreted	Stimulate histamine secretion from basophils, the activation of eosinophils, and the mobilization of monocytes, eosinophils, and memory T helper cells	−403 A/G	Atopic dermatitis	German	[44]
EOTAXIN	Activate eosinophils and Th2, and it might also operate as an indirect negative regulator of neutrophil recruitment.	−426 C/T −384 A/G	IgE levels in atopic dermatitis	Japanese	[45]

Susceptibility gene	Function	Variant	Phenotype	Population	Reference
TLR2 Toll-like receptor 2	It is a pathogen-associated molecular pattern receptors that recognize bacterial and fungal components. Polymorphism might increases the response to infections	Arg753Gln	Severe atopic dermatitis	German	[46]
FCER1B High affinity IgE receptor, beta chain	Upregulation of FceRI expression has been shown in lesional skin of atopic dermatitis, facilitating the uptake and processing of antigen by Langerhans cells and the dermal inflammatory response.	RsaIin2, RsaIex7	Atopic dermatitis	British	[47]
SPINK5 Serine protease inhibitor, Kazal-type 5	Polymorphisms might influence the inhibition of many allergens that are also serine proteases	Glu420Lys	Atopic dermatitis Atopic dermatitis Asthma with atopic dermatitis	British Japanese German	[48] [49] [50]

Susceptibility gene	Function	Variant	Phenotype	Population	Reference
SCCE Stratum corneum chymotryptic enzyme	Play a central role in desquamation by cleaving proteins of the stratum corneum (e.g., corneodesmosin and plakoglobin)	AACCins	Atopic dermatitis	British	[51]
CSTA	Inhibit Der p 1 and Der f 1, major house dust mite cysteine proteases and environmental triggers for AD and asthma.	+344T/C	Atopic dermatitis	British	[52]
COL29A1	Maintain tissue integrity and keratinocyte cohesion. Collagens influence the migration of epidermal antigen presenting cells and T cells. Lack of collagen XXIX may facilitate antigen penetration through the skin and impair the maintenance of cutaneous immune responses.	A36637742 delT	Moderate to severe atopic dermatitis and onset below 2 year atopic dermatitis	European origin	[27]

Susceptibility gene	Function	Variant	Phenotype	Population	Reference
Filaggrin (*FLG*)	Filaggrin aggregate with keratin filaments for condensation of the keratinocyte cytoskeleton and promote compaction of keratinocyte. It degrade into natural moisturising factor, which may contribute to water retention	*FLG* null mutations	Atopic dermatitis	Irish, Scottish, Danish	[18]
			Ichthyosis vulgaris and atopic dermatitis	Japanese	[20]
			Atopic dermatitis	Han Chinese	[21]
			Atopic dermatitis	Singaporean Chinese and European populations	[22]

As for environmental factors, soap and detergents can damage the skin by emulsifying the skin surface lipids resulting in scaling and dryness. Moreover, exogenous proteases, such as, house dust mites that are sources of over 30 different proteins can induce IgE-mediated responses. Proteolysis activity of house dust mite and cockroach allergens induces skin barrier breakdown leading to increased penetration of allergens and pruritus. Staphylococcus aureus releases superantigenic exotoxins that can directly damage the skin barrier and break down corneodesmosomes. They also secrete spingosine deacylase and glycerophospholipids that interfering formation of lipid lamellae [17].

From the above evidences, it has been proposed that impairment of skin barrier, excess of immune responses and environmental factors drive skin barrier defect and induce inflammation in atopic dermatitis.

Biological functions of filaggrin

Profilaggrin is the major constituent of keratohyalin granules that plays an essential role in the terminal differentiation of the epidermis and formation of skin barrier (figure 3). It is encoded by *FLG*, which is located in epidermal differentiation complex on chromosome 1q21. When terminal differentiation of epidermis begins, the keratohyalin granules degranulate and release profilaggrin that is dephosphorylated and proteolysed into filaggrin 10-12 peptides by proteases including matriptase, prostasin and kallikrein 5. The N-terminal domain undergoes nuclear translocation and further degradation into the A and B domains but their functions are unclear. In the stratum corneum, the filaggrin peptides aggregate with keratin filaments for condensation of the keratinocyte cytoskeleton and promote compaction of keratinocyte. Then, the cytoskeleton is cross-linked by transglutaminases and modification by peptidylarginine deiminases to form an insoluble keratin matrix. Cornified cell envelope replaces the keratinocyte cell membrane that forms the skin barrier which prevents water loss and penetration of allergens, and microbes from environment. Subsequently, filaggrin peptides are degraded by a variety of proteases, including caspase 14, into free amino acids, derivatives, urocanic acid and pyrrolidone carboxylic acid, so-called natural moisturising factor, which may also contribute to water retention and UV protection [23].

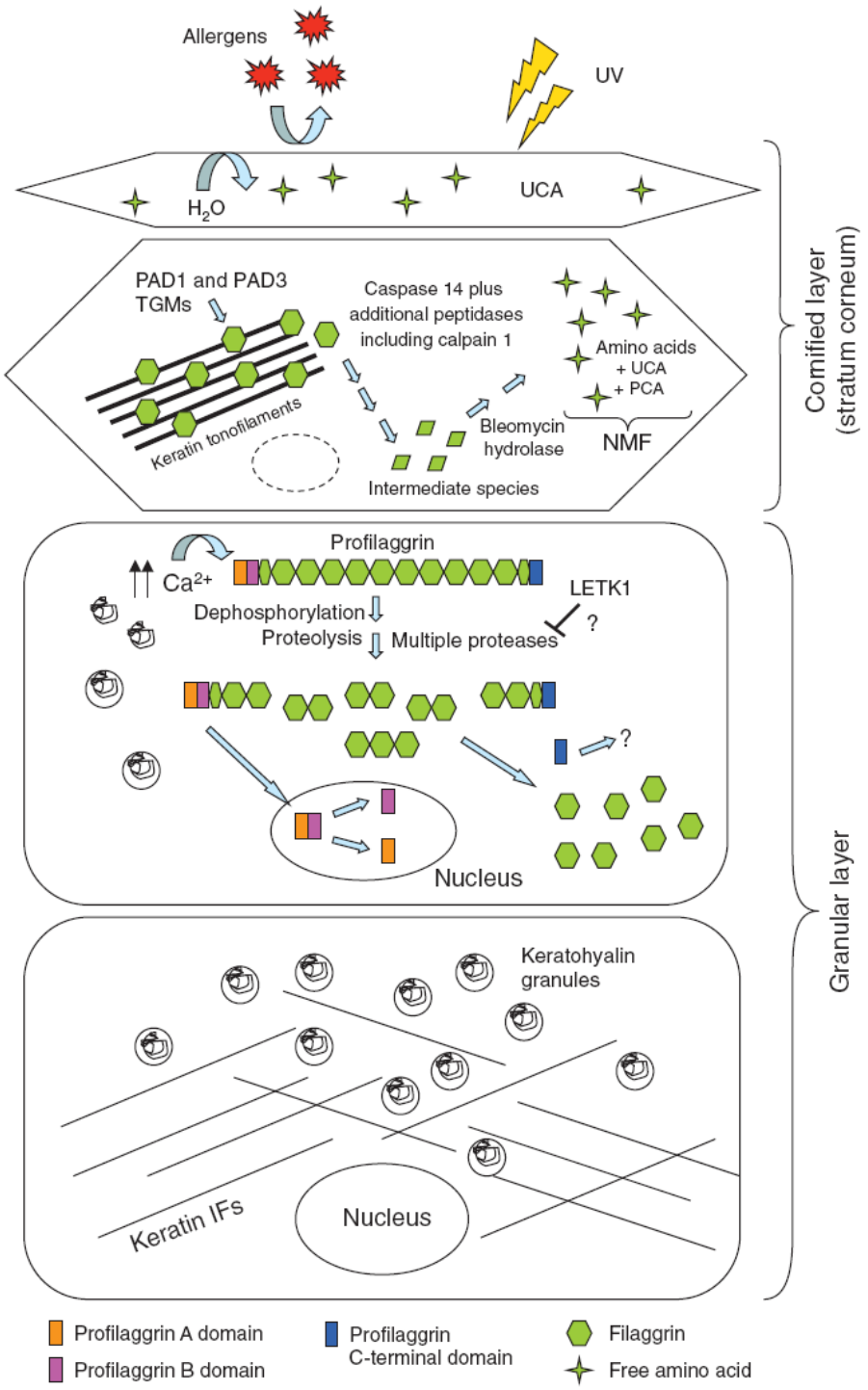


Figure 3. Profilaggrin processing during terminal differentiation of the epidermis [23].

Role of filaggrin in atopic dermatitis pathogenesis

After loss-of-function mutations in *FLG* were identified as a major predisposing factor in atopic dermatitis, it is widely accept that filaggrin deficiency contributes to an impairment of skin barrier function. Immunohistochemical and Western blot analysis showed reduced or absent expression of filaggrin in atopic dermatitis patient who carry *FLG* mutations (figure 4) [19].

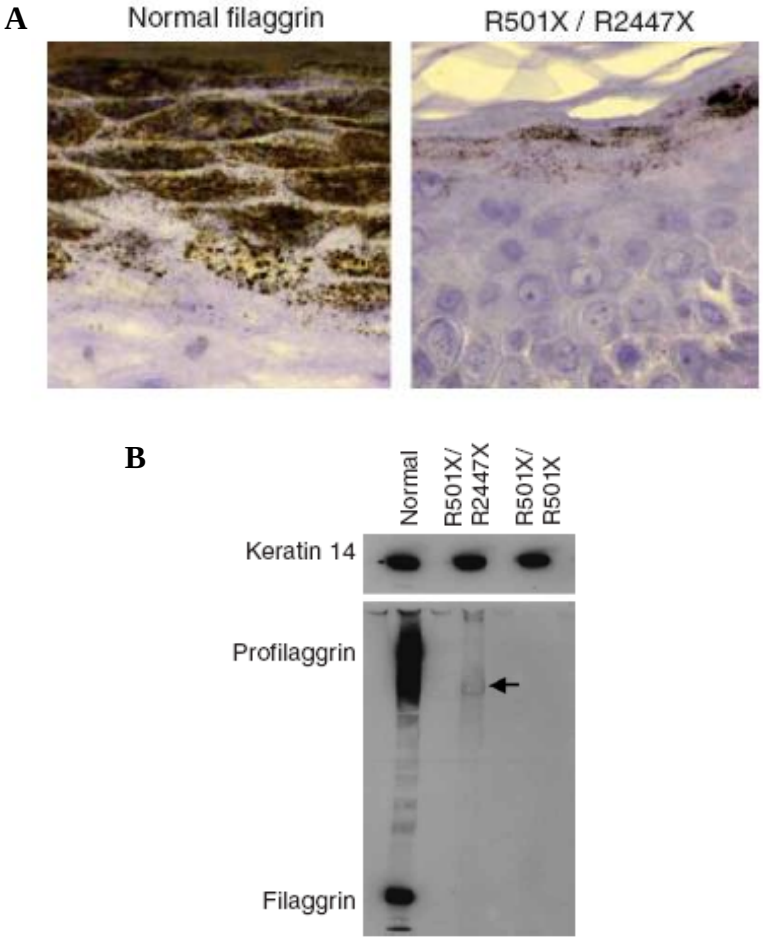


Figure 4. Comparision of filaggrin quantification between *FLG* mutation and normal control. (A) Immunohistochemical staining for filaggrin from skin biopsy of the R501X/R2447X compound heterozygote showed that filaggrin was reduced comparing to the normal control. (B) Western blot analysis in the R501X/R2447X compound heterozygote showed that profilaggrin was decreased and processed filaggrin was absent. The R501X homozygote showed absence of profilaggrin and filaggrin. [19]

Atopic dermatitis patients harboring *FLG* mutations also showed the reduction in levels of natural moisturizing factor derived from filaggrin leading to increased trans-epidermal water loss when compared with normal controls.[53] Moreover, filaggrin deficiency contributes to impairment of keratin intermediate filament organization, maturation and secretion of extracellular lamellar bodies, reduction in corneodesmosome density, decreased tight-junction protein expression and elevation in pH of stratum corneum leading to decreased ability of the corneocytes to retain water and allow penetration of allergens through the defective skin. Elevated pH promotes serine protease activity, inhibits enzymes involved in the synthesis of lipid lamellae and promotes growth of *Staphylococcus aureus*. [54-57]

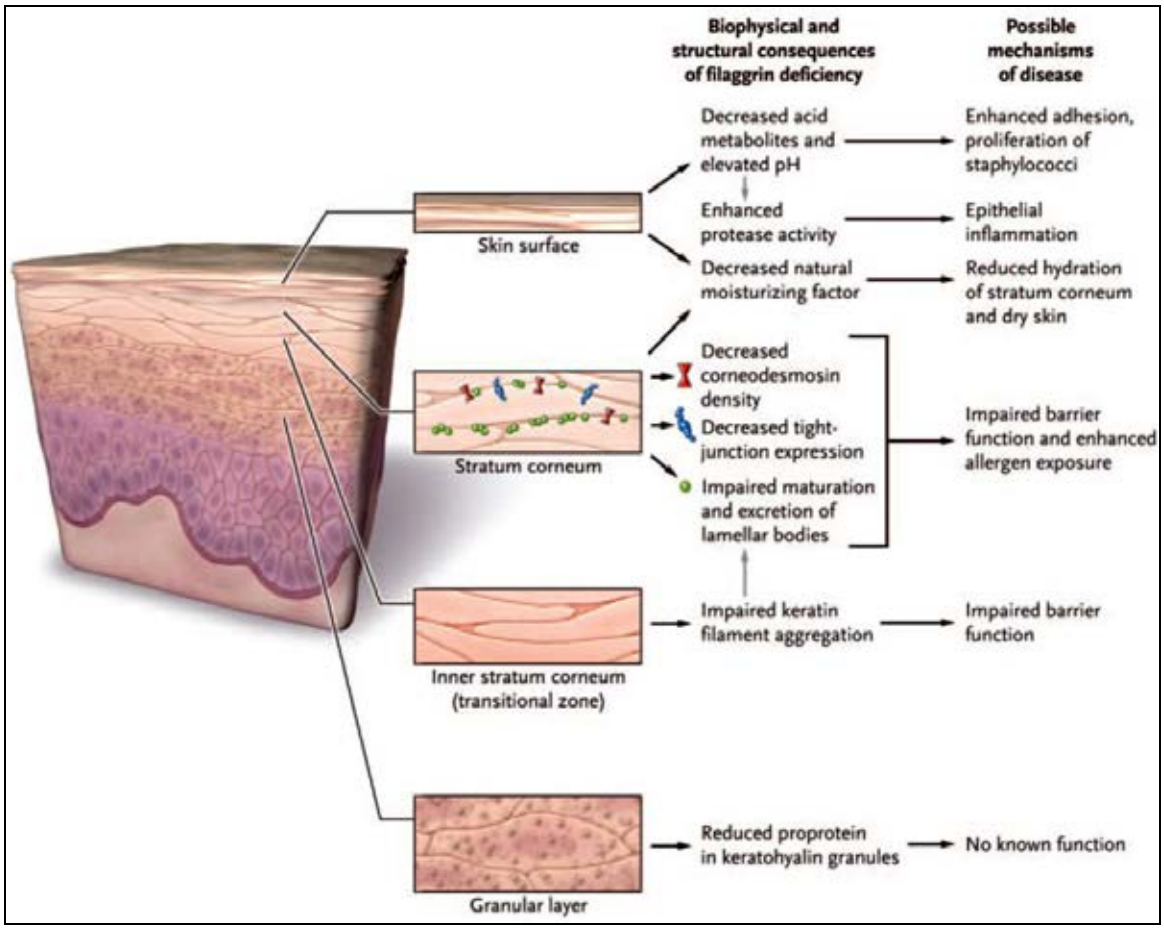


Figure 5. Filaggrin deficiency and possible disease mechanism [58]

In addition, an animal model study in flaky tail mice harboring the 1-bp deletion mutation in murine *Flg* provided evidence suggesting that filaggrin deficiency led to penetration of antigen through the impaired skin barrier and development of skin inflammation.[24] Filaggrin deficiency affects a number of differentiation-specific structural, biophysical, and functional changes within the stratum corneum that are likely to be directly related to disease pathogenesis in atopic dermatitis.

Gene and protein structure of *FLG*

FLG is located on chromosome 1q21. It contains three exons. Exon 1 is noncoding. Exon 2 encodes S100 domain. Both exons are small. Exon 3 is large (~12.7kb) and has highly repetitive DNA sequences and encodes 10–12 filaggrin repeats.[9-10] The 11 repeats allele has duplication of filaggrin repeat 8 or repeat 10. The 12-repeat allele has duplications of both repeat 8 and repeat 10.[19] *FLG* repeats are not only almost 100% similar at the DNA level but also varied in size (figure 6).

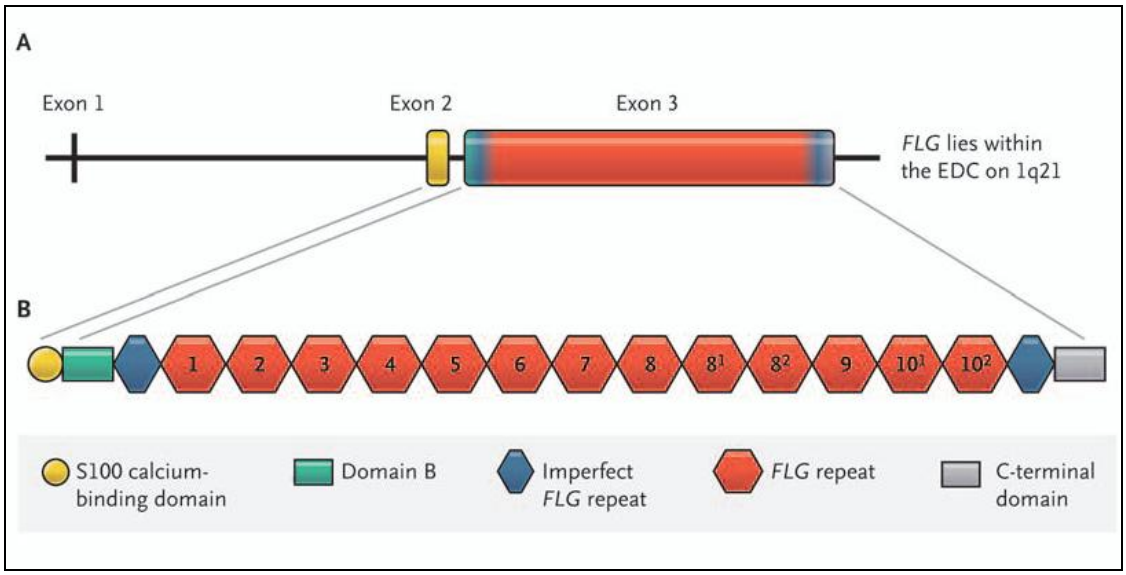


Figure 6. Gene and protein structure of filaggrin. (A) Gene structure of *FLG*. (B) Protein structure of profilaggrin which is encoded by *FLG*. [58]

Previous studies reported over 40 *FLG* mutations in various populations (figure 7) [58]. *FLG* mutations were specific in each population. They were different between Europe and Asia [59].

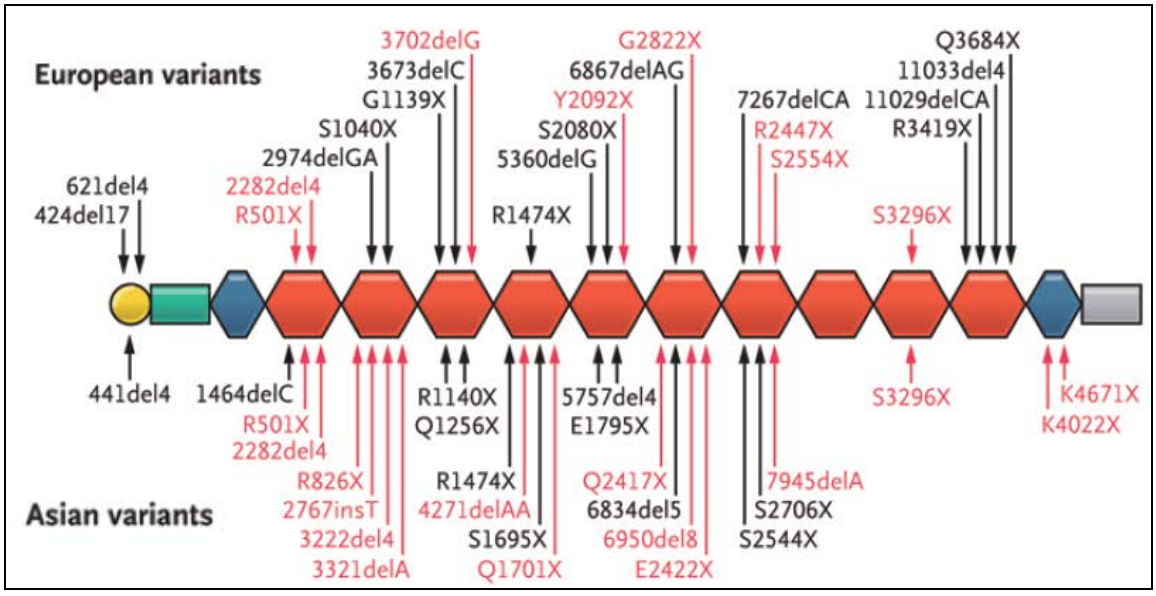


Figure 7. Variations in *FLG* mutations among ethnic groups and other populations. [58]

Gene structure and function of *SLC25A46*

SLC25A46 (solute carrier family 25, member 46) is located on chromosome 5q33.1. It belongs to the SLC25 family of mitochondrial carrier proteins which is a large family of nuclear-encoded transporters embedded in the inner mitochondrial membrane and in a few cases other organelle membranes. The members of this superfamily are involved in numerous metabolic pathways and cell functions. SLC25A46 has no identified substrates and has been reported as an orphan transporter. [60] This gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, mosquito, and *C.elegans* (http://www.ncbi.nlm.nih.gov/gene/91137).

SLC25A46 has 8 exons and encodes solute carrier protein family 25A sub family 46 that expression in skin. Until now, the function of SLC25A46 is still unclear.

Gene structure and function of *TMEM232*

TMEM232 (transmembrane protein 232) is located on chromosome 5q33.1. This gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, and zebrafish (http://www.ncbi.nlm.nih.gov/gene/642987).

TMEM232 has 14 exons and encodes transmembrane protein family 232 that expression in skin. Until now, the function of TMEM232 is still unknown.

CHAPTER III

METHODOLOGY

Subjects and sample collection

1. Subjects

48 unrelated Thai individuals with atopic dermatitis who received treatment at King Chulalongkorn Memorial Hospital were recruited. Inclusion criteria were based on Hanifin and Rajka Diagnostic Criteria for Atopic Dermatitis. [5]

Major criteria:

1. Pruritus
2. Typical morphology and distribution
   Flexural lichenification or linearity in adults
   Facial and extensor involvement in infants and children
3. Chronic or chronically-relapsing dermatitis
4. Personal or family history of atopy (asthma, allergic rhinitis, atopic dermatitis)

Minor criteria:

1. Xerosis
2. Ichthyosis, palmar hyperlinearity, or keratosis pilaris
3. Immediate (type 1) skin-test reactivity
4. Raised serum IgE
5. Early age of onset
6. Tendency toward cutaneous infections (especially *S aureus* and herpes simplex) or impaired cell-mediated immunity
7. Tendency toward non-specific hand or foot dermatitis
8. Nipple eczema
9. Cheilitis
10. Recurrent conjunctivitis
11. Dennie-Morgan infraorbital fold
12. Keratoconus

13. Anterior subcapsular cataracts
14. Orbital darkening
15. Facial pallor or facial erythema
16. Pityriasis alba
17. Anterior neck folds
18. Itch when sweating
19. Intolerance to wool and lipid solvents
20. Perifollicular accentuation
21. Food intolerance
22. Course influenced by environmental or emotional factors
23. White dermographism or delayed blanch

The diagnosis of atopic dermatitis requires the presence of at least three major features and at least three minor features.

2. Controls

50 controls were unaffected ethnically-matched without atopic dermatitis,
ichthyosis vulgaris, psoriasis, atopic disorders, and family history of atopic dermatitis, ichthyosis vulgaris and atopic disorders. DNA from controls was used for mutation screening.

3. Blood collection

After informed consent was obtained, 3 ml of peripheral blood were collected from 48 unrelated Thai individuals with atopic dermatitis in a polypropylene tube with EDTA for DNA extraction.

Genotyping

1. DNA extraction

Genomic DNA was extracted from peripheral leukocytes according to Manual ArchivePure DNA Purification System protocols (5PRIME Inc., Gaithersburg). This procedure was performed as follows:

RBC lysis

1. Pipette 9 ml of RBC Lysis Solution in a 15-ml tube and then
   add 3 ml of well-mixed whole blood. Invert gently to mix.
2. Incubate for 5 minutes at room temperature. Invert gently 3 times during incubation.
3. Centrifuge at 2000 x g for 2 minutes to pellet white blood cells.
4. Pour off the RBC lysis supernatant leaving behind the white blood cell pellet and drain tube for at least 10 seconds on clean, absorbent paper. Less than 200 µl residual liquid should remain.

Cell lysis and protein precipitation

1. Resuspend the white cells by vortexing the tube.
2. Dispense 1 ml of Protein Precipitation Solution into the center of the sample.
3. Dispense 3 ml of Cell Lysis Solution into the center of the sample.
4. Vortex samples at high speed for 20 seconds to complete lysis of white blood cells and precipitate the protein.
5. Centrifuge at 2000 x g for 6 minutes. The precipitated protein should form a tight, dark brown pellet.

DNA precipitation

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a clean 15-ml tube containing 3 ml of 100% isopropanol.

2. Mix the samples by inverting gently together in a tube rack for 50 times to precipitate the DNA.
3. Centrifuge at 2000 x g for 3 minutes. The DNA should be visible as a small white pellet.
4. Carefully pour off the supernatant in one continuous motion. Keep the tube inverted, and drain for 1 minute on clean, absorbent paper.

DNA wash

1. Dispense 3 ml of 70% etanol in to each tube.
2. Centrifuge at 2000 x g for 1 minute to pellet the DNA.
3. Carefully pour off the ethanol in one continuous motion. Keep the tube inverted, and drain for 1 minute on clean, absorbent paper. Pellet may be loose so pour slowly and watch pellet.

DNA hydration

1. Add 250 µl of DNA Hydration Solution (adding 250 µl will give a concentration of 400 µg/ml if the total yield of DNA is 100 µg)
2. Rehydrate the DNA by incubating at 65°C for 1 hour and overnight at room temperature on a rotator.
3. Sample may be centrifuged briefly and then transferred to a storage tube.
4. Store DNA at 4°C

2. DNA amplification by Polymerase Chain Reaction (PCR)

Mutation analysis was performed in three candidate genes, *FLG*, *SLC25A46*, and *TMEM232.*

2.1 *FLG* amplification

PCR of *FLG* was performed with repeated-specific primers as previously described [19] with some modifications. The PCR primers used for *FLG* amplification are shown in table 3.

Table 3. PCR primers and product size of *FLG* [19]

***FLG* exon**	**Primer pair**	**Product size (bp)**	**Annealing temperature (°C)**
1	5’ CGT GAG GAA GCT GGG AAG TA 3’ 5’ TTA TGC CCT CAT TTT CCT TCT 3’	381	60
2	5’ CTA CTA AGT CCA GCT GTA AGT G 3’ 5’ GCT CTA TCT TTG GTC TTG TCA G 3’	431	60
Exon 3 repeat	**Primer pair**	**Product size (bp)**	**Annealing temperature (°C)**
1-3	5’ GCT GAT AAT GTG ATT CTG TCT G 3’ 5’ GAC CCC GAT GAT TGT TCC TGT 3’	3697	61
3-5	5’ GCA AGC AGA CAA ACT CGT AAG 3’ 5’ ACA TCA GAC CTT TCC TGG GAC 3’	1916	61
4-7	5’ GAC AAG ATT CAT CTG TAG TCG 3’ 5’ CTG GCT AAA ACT GGA TCC CCA 3’	2609	64
7-8	5’ CCA CAC GTG GCC GGT CAG CA 3’ 5’ CTA CCG AAT GCT CGT GGT GGT 3’	1224	64
7-10	5’ CCC AGG ACA AGC AGG AAC T 3’ 5’ GCT TCA TGG TGA TGC GAC CA 3’	2858/ 3830	62
9-10	5’ GAA ACG TCT GGA CAT TCA GGA 3’ 5’ GCT TCA TGG TGA TGC GAC CA 3’	1367	61
10	5’ GCC CAT GGG CGG ACC AGG A 3’ 5’ CTG CAC TAC CAT AGC TGC C 3’	1753/ 2728	62
End (partial repeat)	5’ CTA GTA CCG CTA AGG AAC ATG G 3’ 5’ TGG CTC CTT CGA TAT TTC TGA 3’	781	58

The PCR conditions of *FLG* are shown in table 4 and 5. The PCR amplifications were performed in Mastercycler pro S (Eppendorf, Germany). As for PCR amplification of repeat 10, it was performed in a 25-µl reaction volume containing 100 ng of genomic DNA, 1.5 mM MgCl2, 0.4 µM forward primer, 0.4 µM reverse primer, 200 µM of each dNTP, 4% DMSO and 1.3 units of Expand High Fidelity enzyme mix (Roche).[19] PCR conditions were as follows: 94˚C for 2 minutes, 10 cycles of 94˚C for 15 seconds, 62˚C for 30 seconds, 72˚C for 2 minutes, followed by 20 cycles of 94˚C for 15 seconds, 62˚C for 30 seconds, 72˚C for 2 minutes plus 5 seconds of cycle elongation for each successive cycle, and final extension at 72˚C for 7 minutes. The *FLG* repeats 7–10 were amplified in a 25- µl reaction volume containing 100 ng of genomic DNA, 0.1 µM forward primer, 0.1 µM reverse primer, and Platinum Taq PCR SuperMix (Invitrogen). PCR conditions were as follows: 94˚C for 5 minutes, 35 cycles of 94˚C for 30 seconds, 62˚C for 30 seconds, 72˚C for 4 minutes and final extension at 72˚C for 7 minutes. Because of size variants in *FLG*, PCR amplification of repeats 7-10 and repeat 10 showed two different sizes of bands in individuals harboring the heterozygous alleles for 10 and 11 repeats. Targeted bands were cut and extracted by using QIAquick® gel extraction kit (QIAGEN).

Table 4. PCR components for *FLG* amplification

Component	**Exon 3 repeat 1-3**	**Exon 3 repeat 3-5**	**Exon 3 repeat 4-7**	**Exon 3 repeat 7-8**
Distilled water (µl)	Up to 50	Up to 50	Up to 50	Up to 20
10x PCR Buffer with 15 mM $MgCl_2$	1x (1.5 mM $MgCl_2$)	1x (1.5 mM $MgCl_2$)	1x (1.5 mM $MgCl_2$)	1x (1.5 mM $MgCl_2$)
25 mM $MgCl_2$	-	0.5 mM	-	0.3 mM
5x Q-Solution	-	1x	-	1x
dNTP	200 µM	200 µM	200 µM	200 µM
Forward primer	0.2 µM	0.2 µM	0.2 µM	0.15 µM
Reverse primer	0.2 µM	0.2 µM	0.2 µM	0.15 µM
HotStarTaq DNA Polymerase	2.5 U/µl	2.5 U/µl	2.5 U/µl	1 U/µl
Genomic DNA	200	200	200	100
Total volume (µl)	50	50	50	20
Component	**Exon 1**	**Exon 2**	**Exon 3 end**	**Exon 3 repeat 9-10**
Distilled water (µl)	Up to 25	Up to 25	Up to 25	Up to 20
10x *Taq* Buffer with $(NH_4)_2SO_4$	1x	1x	1x	1x (1.5 mM $MgCl_2$)
25 mM $MgCl_2$	1.5 mM	1.8 mM	1.7 mM	-
DMSO	4%	4%	4%	1x
dNTP	200 µM	200 µM	200 µM	200 µM
Forward primer	0.12 µM	0.12 µM	0.2 µM	0.2 µM
Reverse primer	0.12 µM	0.12 µM	0.2 µM	0.2 µM
Taq DNA Polymerase	0.5 U/µl	0.5 U/µl	0.5 U/µl	HotStarTaq DNA Polymerase 1 U/µl
Genomic DNA	100	100	100	100
Total volume (µl)	25	25	25	20

Table 5. PCR conditions for *FLG* amplification

Step	**Exon 1**			**Exon 2**		
	Temperature	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**
Initial denaturation	94°C	3 min	1	94°C	3 min	1
Denaturation Annealing Extension	94°C 60°C 72°C	30 sec 45 sec 45 sec	35	94°C 60°C 72°C	30 sec 45 sec 45 sec	35
Final extension	72°C	7 min	1	72°C	7 min	1
Step	**Exon 3 repeat 1-3**			**Exon 3 repeat 3-5**		
	Temperature	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**
Initial denaturation	95°C	15 min	1	95°C	15 min	1
Denaturation Annealing Extension	94°C 61°C 72°C	1 min 45 sec 3 min	35	94°C 61°C 72°C	1 min 45 sec 1.40 min	35
Final extension	72°C	10 min	1	72°C	10 min	1
Step	**Exon 3 repeat 4-7**			**Exon 3 repeat 7-8**		
	Temperature	**Time**	**Cycle**	**Temperature**	**Time**	**Cycle**
Initial denaturation	95°C	15 min	1	95°C	15 min	1
Denaturation Annealing Extension	94°C 62°C 72°C	1 min 45 sec 3 min	35	94°C 64°C 72°C	1 min 45 sec 1.20 min	35
Final extension	72°C	10 min	1	72°C	10 min	1
Step	**Exon 3 repeat 9-10**			**Exon 3 end**		
	Temperature	**Time**	**Cycle**	**Temperature**	**Time**	**cycle**
Initial denaturation	95°C	15 min	1	94°C	5 min	1
Denaturation Annealing Extension	94°C 61°C 72°C	1 min 45 sec 1.30 min	35	94°C 58°C 72°C	30 sec 45 sec 50 sec	35
Final extension	72°C	10 min	1	72°C	7 min	1

2.2 *SLC25A46* amplification

The entire coding region of *SLC25A46* was amplified with specific primers and condition as shown in table 6, 7 and 8.

Table 6. PCR primers for amplification of *SLC25A46*

Exon	Sequence	Product size (bp)	Annealing temperature (°C)
1	5’ TTA ATG GTT GCC GGA AGA GG 3’ 5’ GGA TGA GAA GGA ATA GGA GG 3’	581	56
2	5’ CCA GCA GAG TCC TTC ATT CA 3’ 5’ TGA CAC GTC AGT TAC ATG GC 3’	665	64
3	5’ TGC ATG ATT CTT CAG GCA AG 3’ 5’ CCA AAG TTA TTT AGG ATC CTC 3’	337	60
4	5’ CCA GTC AGT TGC CAA GTA CA 3’ 5’ CAC AGC TCT TTG TGG TTT TGC 3’	648	64
5	5’ ACA GGA CTA GTA GGG AGT TG 3’ 5’ GAC AAG GGT TGA AAG CTA CC 3’	557	60
6	5’ CTC TTG CTT CTC TGC CAG CC 3’ 5’ GTC CCA CCA ACA TTT CAC TGC 3’	423	60
7	5’ GCT ATG TGT AGG TAG ATG TCC 3’ 5’ ATA CAG ACA AGC AAC GTG CG 3’	488	60
8	5’ TCA TGG ATG TTT CCC TCT TCA G3’ 5’ AAT CAG TAT GGG CTT TAG TAC C3’	836	64

Table 7. PCR components for amplification of *SLC25A46*

Component	Exon 1-8
Distilled water (µl)	Up to 20
10X *Taq* Buffer with $(NH_4)_2SO_4$	1X
25 mM $MgCl_2$	1.5 mM
dNTP	200 µM
10 µM forward primer	0.2 µM
10 µM reverse primer	0.2 µM
5 U/µl *Taq* DNA polymerase	0.5 U/µl
Genomic DNA	100 ng
Total volume (µl)	20

Table 8. PCR conditions for *SLC25A46* amplification

Step	**Exon 1**	**Exon 2**	**Exon 3**	**Exon 4**
Initial denaturation	94°C/5 min	94°C/5 min	94°C/5 min	94°C/5 min
PCR cycle	35	35	35	35
Denaturation	94°C/30 sec	94°C/30 sec	94°C/30 sec	94°C/30 sec
Annealing	56°C/30 sec	64°C /30 sec	60°C /30 sec	64°C /30 sec
Extension	72°C	72°C	72°C	72°C
Final extension	72°C/7 min	72°C/7 min	72°C/7 min	72°C/7 min
Step	**Exon 5**	**Exon 6**	**Exon 7**	**Exon 8**
Initial denaturation	94°C/5 min	94°C/5 min	94°C/5 min	94°C/5 min
PCR cycle	35	35	35	35
Denaturation	94°C	94°C	94°C	94°C
Annealing	60°C /30 sec	60°C /30 sec	60°C /30 sec	64°C /30 sec
Extension	72°C	72°C	72°C	72°C
Final extension	72°C/7 min	72°C/7 min	72°C/7 min	72°C/7 min

2.3 *TMEM232* amplification

The entire coding region of *TMEM232* was amplified with specific primers and conditions as shown in table 9, 10 and 11.

Table 9. PCR primers for amplification of *TMEM232*

Exon	**Sequence**	**Product size (bp)**	**Annealing temperature (°C)**
1	5’ TGG CTT CTT GCA GGA TAT GC 3’ 5’ CCT GTG AGG ATC TTT GTG GG 3’	382	60
2	5’ TCT TAG CTA CCA TGC CAT GC 3’ 5’ TCT TTG GTT AGA GAA CTA TGG G 3’	518	60
3	5’ ACA GGA TCA GTC CAG GAG GC 3’ 5’ AAC GTT TTG CTA TGG GCT GC 3’	508	62
4	5’ TAC AAC TCT GGC CAC CAA GC 3’ 5’ GGA GGC ATA AAT GAG GAG TG 3’	682	64
5	5’ GGG CCT GCT CAA GTT TAA GG 3’ 5’ AGT TTC CCA AAT TGG GCA GC 3’	735	66
6	5’ ACT CAC TTA AGG GGC ATG AC 3’ 5’ GTG CTC TTC AAC ACT GCA CC 3’	536	60
7	5’ GGA AAC TAG CAG TTT CTG CAT 3’ 5’ GGA ACT GTG CAT ATG GTT GC 3’	463	60
8	5’ GTC AGG GAG AAC TAA GTC AC 3’ 5’ CCA TTG CCT TTG GAA CTG AG 3’	606	62
9	5’ GAC ATG CAT TTC CTC TGA CC 3’ 5’ GCA TAC TAT ATG CGC TAA TAC G 3’	327	58
10	5’ AGT CTA CTC ATG GTA AAC CC 3’ 5’ ACA GAG TGA CAC CTG GCC TC 3’	572	60
11	5’ GGG TCA GAA ATT TCC ACA GC 3’ 5’ CGT CTA GAG CTC AAC TCT CC 3’	511	62

12	5’ TGA AAT TCT TAC ACG GCA CG 3’ 5’ GAG AAG GGA GGA ACT AGC TC 3’	709	62
13	5’ AGT GAC CCA AGA TGG CTT AC 3’ 5’ TGG CAA ACC AAT CAT GGC TC 3’	406	60
14	5’ GCT TTG TGG GAC TTA CAT GC 3’ 5’ TCT AAG ATG ACC AGC CCT CC 3’	928	62

Table 10. PCR components for *TMEM232* amplification

Components	**Exon 1-8**
Distilled water (µl)	Up to 20
10X *Taq* Buffer with $(NH_4)_2SO_4$	1X
25 mM $MgCl_2$	1.5 mM
dNTP	200 µM
10 µM forward primer	0.2 µM
10 µM reverse primer	0.2 µM
5 U/µl *Taq* DNA polymerase	0.5 U/µl
Genomic DNA	100 ng
Total volume (µl)	20

Table 11. PCR conditions for *TMEM232* amplification

Step	**Exon 1**	**Exon 2**	**Exon 3**	**Exon 4**	**Exon 5**	**Exon 6**	**Exon 7**
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35	35	35	35	35	35	35
Denaturation	94°C/30 s	94°C/30 s	94°C/30 s	94°C/30 s	94°C	94°C	94°C
Annealing	56°C/30 s	64°C /30 s	60°C /30 s	64°C /30 s	66°C /30 s	60°C /30 s	60°C /30 s
Extension	72°C/30 s	72°C/35 s	72°C/35 s	72°C/40 s	72°C/45 s	72°C/35 s	72°C/30 s
Final extension	72°C/7min	72°C/7min	72°C/7min	72°C/7min	72°C/7min	72°C/7min	72°C/7min
Step	**Exon 8**	**Exon 9**	**Exon 10**	**Exon 11**	**Exon 12**	**Exon 13**	**Exon 14**
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35	35	35	35	35	35	35
Denaturation	94°C/30 s	94°C/30 s	94°C/30 s	94°C/30 s	94°C/30 s	94°C/30 s	94°C/30 s
Annealing	62°C /30 s	58°C /30 s	60°C /30 s	62°C /30 s	62°C /30 s	60°C /30 s	62°C /30 s
Extension	72°C/40 s	72°C/30 s	72°C/35 s	72°C/35 s	72°C/45 s	72°C/30 s	72°C/60 s
Final extension	72°C/7min	72°C/7min	72°C/7min	72°C/7min	72°C/7min	72°C/7min	72°C/7min

3. Agarose gel electrophoresis

The PCR products were verified for correct size on an ethidium bromide stained 0.8-1.2% agarose gel. Then, PCR products were purified and treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation and sent for direct sequencing (Macrogen Inc., Seoul, Korea)

4. DNA sequencing

The PCR products were sent for direct sequencing at the Macrogen Inc., (Seoul, Korea). Primers used for *FLG* sequencing are shown in Table 12.

Table 12. Primers for sequencing of *FLG*

Exon	Primer name	Primer sequences
1	FLG-ex1-F	5' CGT GAG GAA GCT GGG AAG TA 3'
2	FLG-ex2-F	5' CTA CTA AGT CCA GCT GTA AGT G 3'
Exon 3 repeat	**Primer name**	**Primer sequences**
1-3	FLG1-3F	5' GCT GAT AAT GTG ATT CTG TCT G 3'
	FLGE3F1	5' GAC AAT AGG AAG AGG CTA AG 3'
	FLGE3F2	5' CTT CCT CTC GTG GAC AGA CT 3'
	FLGE3F4	5' CTC GTC ACA CAC AGA ATT CC 3'
	FLG1-3inF2	5' CAG GCC AGG GAC AAT CAG AG 3'
3-5	FLG3-5F	5' GCA AGC AGA CAA ACT CGT AAG 3'
	FLGE3F6	5' AGA CAC TCA GGC ATT GGG CA 3'
	FLG4-7F	5' GAC AAG ATT CAT CTG TAG TCG 3'
4-7	FLG4-7F	5' GAC AAG ATT CAT CTG TAG TCG 3'
	FLGE3F8	5' AAA CACGTAATGA GAAACAA 3'
	FLG4-7R	5' CTG GCT AAA ACT GGA TCC CCA 3'
7-8	FLG7-8F	5' CCA CAC GTG GCC GGT CAG CA 3'
	FLG7-10F	5' CCC AGG ACA AGC AGG AAC T 3'
7-10 (8)	FLG7-10F	5' CCC AGG ACA AGC AGG AAC T 3'
	FLG7-10inR1	5' CGA ATG GTG TCC TGA CCG TA 3'
7-10 (8.1+8.2)	FLG7-10F	5' CCC AGG ACA AGC AGG AAC T 3'
	FLG-Seq8.1F1	5' TGGGCAGTCAGGATCCAGAC 3'
	FLG7-10inR1	5' CGA ATG GTG TCC TGA CCG TA 3'
9-10	FLG9-10R2	5' GCA GAT GAA GCT TGT CCA CG 3'
	FLG9-10R	5' GCT TCA TGG TGA TGC GAC CA 3'
10	FLG10F	5' GCC CAT GGG CGG ACC AGG A 3'
	FLG10R	5' CTG CAC TAC CAT AGC TGC C 3'
10.1+10.2	FLG10F	5' GCC CAT GGG CGG ACC AGG A 3'
	FLG10inF10	5' TCC ACCCATGGAC AGTCTG 3'
	FLG10R	5' CTG CAC TAC CAT AGC TGC C 3'
end	endR	5' TGG CTC CTT CGA TAT TTC TGA 3'

Sequence analysis and mutation prediction

The DNA sequence was analyzed by Sequencher (version 5.0; Gene Codes Corporation, Ann Arbor, MI) and compared with NCBI Reference Sequence: NG_016190.1. Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) online softwares were used to predict the possible impact of all mutations on the protein function.

Mutation confirmation and screening

The mutations found in *FLG* were confirmed and screened in 100 unaffected ethnically-matched chromosomes by Pyrosequencing of a short PCR fragment and direct-sequencing. Restriction enzyme digestion was not performed because mutations did not generate restriction sites different from the wild-type sequence. A short PCR fragment comprising the c.4665delG was amplified by using specific forward primer (5'AGAAGTGCAAGCAGGCAAAC3'F) and reverse primer (5'GTGTCCCTCACTGT
CCCTGT 3'R-biotin). PCR fragment length is 198 bp. Typically, 150 ng of DNA was amplified in a 20-µl reaction containing 2.5 mM MgCl2, 0.6 µM forward primer, 0.6 µM reverse primer, 200 µM of each dNTP, and 1 units U *Taq* DNA polymerase (Fermentas). PCR conditions were as follows: 94°C for 5 min followed by 35 cycles of 94 °C for 30 sec, 65°C for 30 sec and 72 °C for 20 sec and a final extension at 72 °C for 5 min. Sequencing primers for Pyrosequencing is 5'AGAAATGAGGAACAATCAGGAGAC3' Analysis mode used for quantification of diferrent alleles was selected. The dispensation order is GCTCCAG[G]CACTCA. For Pyrosequencing protocol, we followed PyroMark® Q24 User Manual, QIAGEN.

As for c.1576G>T (p.G526X), c.11944C>T (p.R3982X), and c.5717C>A (p.S1906X) that present in *FLG* fragment 1-3, 10, and 4-7, respectively were confirmed and screened by PCR-sequencing using primers and PCR conditions from the above descript in Table 3 and 4.

Pyrosequencing protocol

1. Setting up a run

Setting up an assay select ―New Assay" followed by the desired assay type AQ from the type or paste the ―Sequence to Analyze" (GCTCCAGGCACTCA) and then click the ―Generate Dispensation Order"

1. In the shortcut browser, right-click the folder you want to place the run file in and select ―New Run" from the context menu.
2. Enter the file name and press ―Enter".
3. Select ―Instrument Method"
4. Add an assay to each well used, e.g., drag an assay from the shortcut browser to a well or a selection of wells. A well is colored according t the assay loaded into the well.
5. To enter a sample ID or note, select the cell and enter the text. A selected cell is highlighted with a blue background color.
6. Click in the toolbar.
7. Print a list of required volumes of reagents and the plate setup; select ―Plate Run Information" from the ―Tools" menu and, when the report appears, click .
8. Close the run file and copy it to one of the USB sticks supplied.

2. Sample preparation

Samples to be analyzed using the PyroMark Q24 Instrument should be prepared according to the instructions below. The following equipment and reagents are required for sample preparation. All reagents and solutions should be at room temperature (15–25°C) before starting. All steps are performed at room temperature unless otherwise stated.

Equipment and reagents to be supplied by the user

- PyroMark Q24 Vacuum Workstation
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80°C
- PyroMark Q24 Plate

- 24-well PCR plate or strips
- Strip caps
- PyroMark Q24 Cartridge
- Streptavidin Sepharose High Performance (34 µm, 5 ml, GE Healthcare)
- Sequencing primer
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- Ethanol (70%)
- PyroMark Binding Buffer
- PyroMark Denaturation Solution
- PyroMark Wash Buffer concentrate
- PyroMark Annealing Buffer

DNA amplification

Amplify the DNA to be analyzed by PCR using one of the primers biotinylated. forward primer (5'AGAAGTGCAAGCAGGCAAAC3'F) and reverse primer (5'GTGTCCCTCACTGTCCCTGT 3'R-biotin) Immobilizing the PCR product to beads Biotinylated PCR products are immobilized on streptavidincoated Sepharose beads (Streptavidin Sepharose High Performance, GE Healthcare).

1. Gently shake the bottle with streptavidin-coated Sepharose beads from side to side until a homogenous solution is obtained.
2. Mix the total amount of streptavidin-coated Sepharose beads (2 µl per sample) and Binding Buffer (40 µl per sample) in a tube. Add high-purity water to a total volume of 80 µl per well — including the PCR product to be added in step 4. The amount of water depends on the amount of PCR product used. For example: If using 15 µl of PCR

product, 2 µl of beads, and 40 µl of Binding Buffer, 23 µl of high-purity water must be added.

3. Add the solution prepared in step 2 to a 24-well PCR plate or strips.
4. Add 5–20 µl of a well-optimized, biotinylated PCR product to each well of the PCR plate (or strips) according to the plate setup. Note: The total volume per well should be 80 µl.
5. Seal the PCR plate (or the strips) using strip caps. Ensure that no leakage is possible between the wells.
6. Agitate the PCR plate (or strips) constantly for at least 5–10 min using a mixer (1400 rpm).

Separation of DNA strands and release of samples into the PyroMark

Q24 Plate

Prewarm one of the supplied PyroMark Q24 Plate Holders

1. Ensure that the PyroMark Q24 Vacuum Workstation has been assembled correctly and securely. The mains plug should be easily accessible in case the vacuum pump needs to be disconnected quickly from the mains power.
2. Fill five separate troughs supplied with the PyroMark Q24 Vacuum Workstation as follows:
    - Approximately 50 ml ethanol (70%)
    - Approximately 40 ml Denaturation Solution
    - Approximately 50 ml 1x Wash Buffer
    - Approximately 50 ml high-purity water
    - Approximately 70 ml high-purity water

    A suggested setup is shown below. Refill the troughs to these levels whenever necessary.
3. Switch on the vacuum pump.
4. Apply vacuum to the tool by opening the vacuum switch.

5. Wash the filter probes by lowering the probes into high purity water. Flush the probes with 70 ml high purity water.
6. Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.
7. Refill with 70 ml high-purity water.
8. Dilute the sequencing primer to 0.3 µM in Annealing Buffer. Add 25 µl of the solution to each well of a PyroMark Q24 Plate that is to be used.
9. Immediately after immobilization, place the PCR plate (or the strips) and PyroMark Q24 Plate on the worktable. Ensure that the plate is in the same orientation as when the samples were loaded.
10. Apply vacuum to the tool by opening the vacuum switch.
11. Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the filter probes in place for 15 s. Take care when picking up the tool.
12. Ensure that all liquid is aspirated from the wells and that all beads have been captured onto the filter probe tips.
13. Transfer the tool to the trough containing 70% ethanol. Flush the filter probes for 5 s.
14. Transfer the tool to the trough containing Denaturation Solution. Flush the filter probes for 5 s.
15. Transfer the tool to the trough containing Wash Buffer. Flush the filter probes for 10 s.
16. Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.
17. While holding the tool over the PyroMark Q24 Plate, close the vacuum switch on the tool.
18. Release the beads in the plate containing sequencing primer, by shaking the tool gently from side to side.

19. With the vacuum switch closed, transfer the tool to the trough containing high-purity water and agitate the tool for 10 s.
20. Wash the filter probes by lowering the probes into high purity water and applying vacuum. Flush the filter probes with 70 ml high-purity water.
21. Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.
22. Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.
23. Turn off the vacuum pump.
24. At the end of a working day, liquid waste and any remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage

Annealing of sequencing primer to samples

1. Heat the PyroMark Q24 Plate containing the samples at 80°C for 2 min using the PyroMark Q24 Plate Holder (two are supplied with the vacuum workstation) and a heating block.
2. Remove the plate from the plate holder and allow the samples cool to room temperature (15–25°C) for at least 5 min. The plate can now be processed in the PyroMark Q24 Instrument.

3. Preparation of PyroMark Gold Q24 Reagents

1. Open the PyroMark Gold Q24 Reagents box and remove the vials containing freeze-dried enzyme and substrate mixtures, and the tubes containing nucleotides.
2. Reconstitute the volumes of reagents required and fill PyroMark Q24 Cartridge according to the handbook supplied with the

reagents. The required volumes of reagents are listed in the "Pre Run Information" report

4. Processing a run on the PyroMark Q24 Instrument

1. Starting the run
2. Load the reagent cartridge and the plate:
3. When the instrument is not processing, open the instrument lid.
4. Open the cartridge gate and insert the filled reagent cartridge with the label facing out. Push the cartridge in fully and then push it down.
5. Ensure the cartridge is properly inserted, with the line visible in front of the cartridge, and close the gate.
6. Open the plate-holding frame and place the plate on the heating block inside the instrument.
7. Close the plate-holding frame and the instrument lid.

5. Select the run file and start the run:

1. Insert the USB stick containing the run file into the USB port at the front of the instrument.
2. Select "Run" in the main menu and press "OK".
3. Select the run file using the up and down screen buttons. To view the contents of a folder, select the folder and press "Select". To go back to the previous view, press "Back".
4. When the run file is selected, press "Select" to start the run.

6. Analyzing the run

Detailed instructions for analyzing the run are available in the *PyroMark Q24 Software User Guide* (press the "F1" key when in PyroMark Q24 Software).

1. Move the processed run file from the USB stick to a computer running PyroMark Q24 Software.
2. Open the run file by double-clicking the run file ( ) in the

shortcut browser. If several assay types are included, select analysis mode in the dialog box that opens. To add a shortcut to a file or folder in the shortcut browser, click ―Add File Shortcut" or ―Add Folder Shortcut".

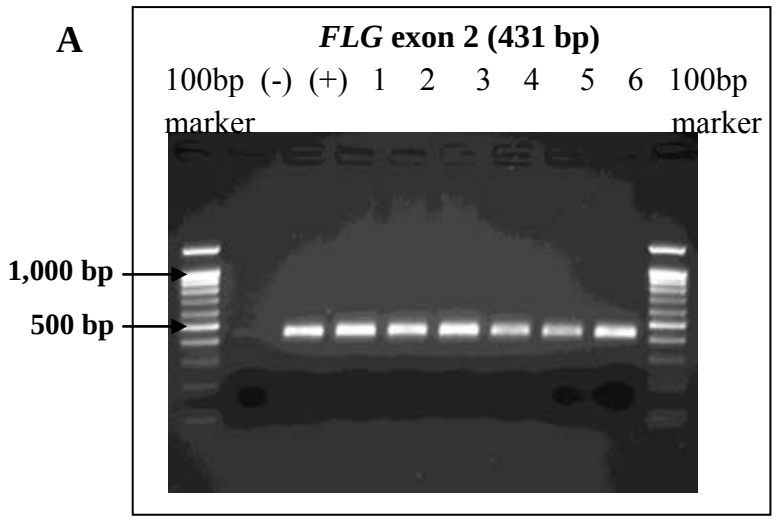
3. In the ―Overview" tab, either analyze all wells or a selection of wells with a valid analysis setup for the AQ analysis mode. The allele frequencies are displayed in Pyrogram, for example and (InDel). The quality assessment is displayed by the background color of the result.

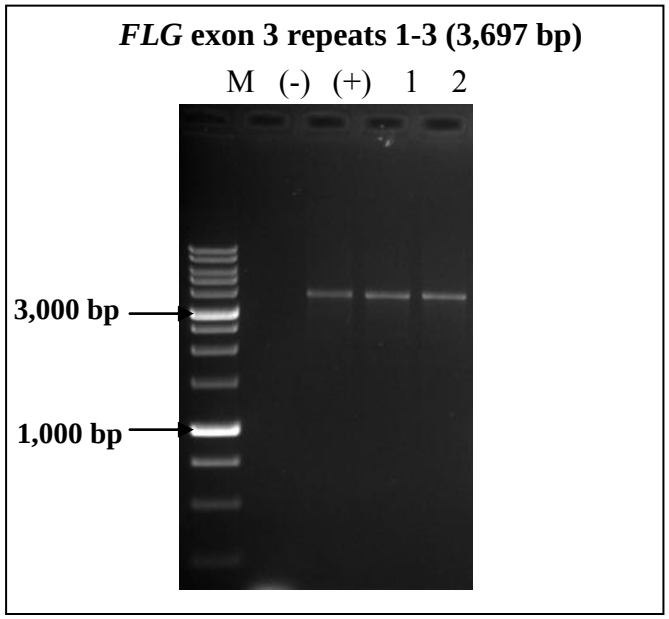
CHAPTER IV

RESULTS

Polymerase Chain Reaction (PCR) amplification of *FLG*

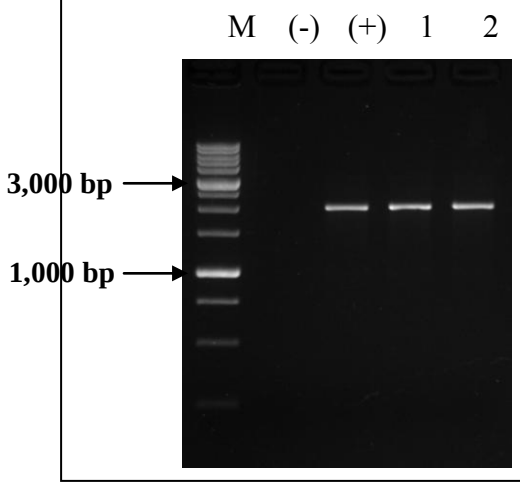
We performed PCR-sequencing of the entire coding regions of *FLG* in 48 unrelated atopic dermatitis patients. Figure 8A-I showed examples of the results after agarose gel electrophoresis of the PCR products. Each PCR reaction produced the correct band. Non-specific bands were not observed.





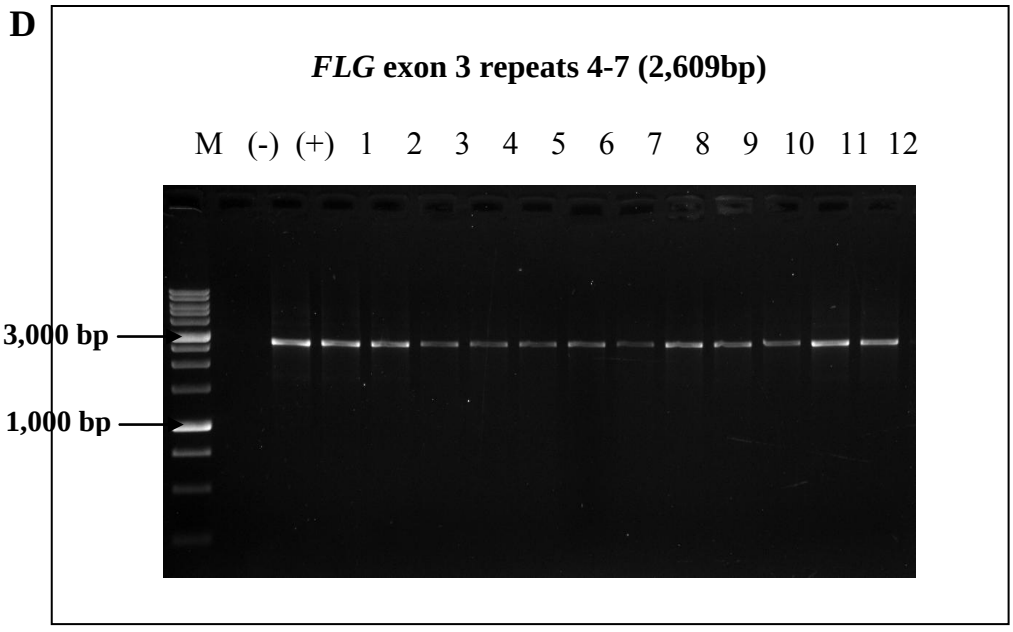
C

***FLG* exon 3 repeats 3-5 (1,916 bp)**

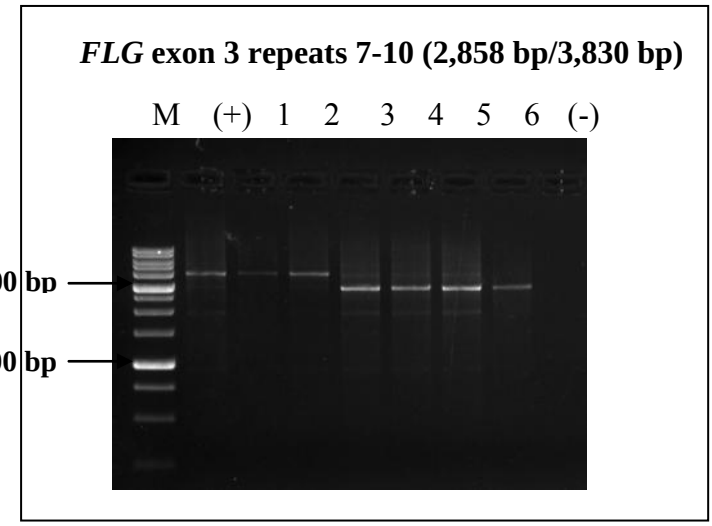


D

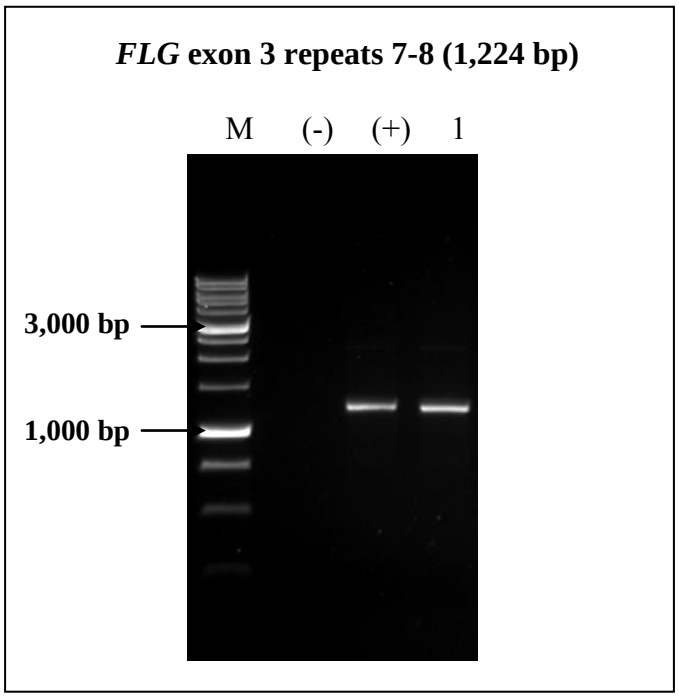
***FLG* exon 3 repeats 4-7 (2,609bp)**



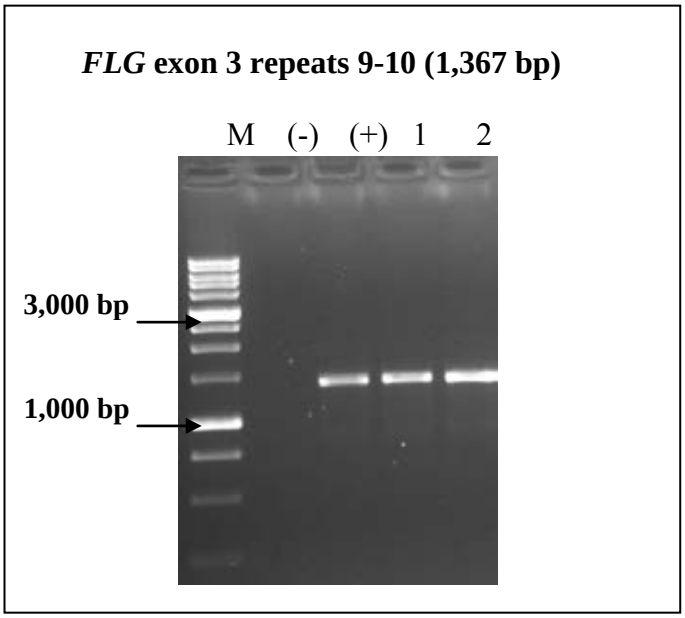
E


FLG exon 3 repeats 7-10 (2,858 bp/3,830 bp)
M (+) 1 2 3 4 5 6 (-)
3,000 bp
1,000 bp

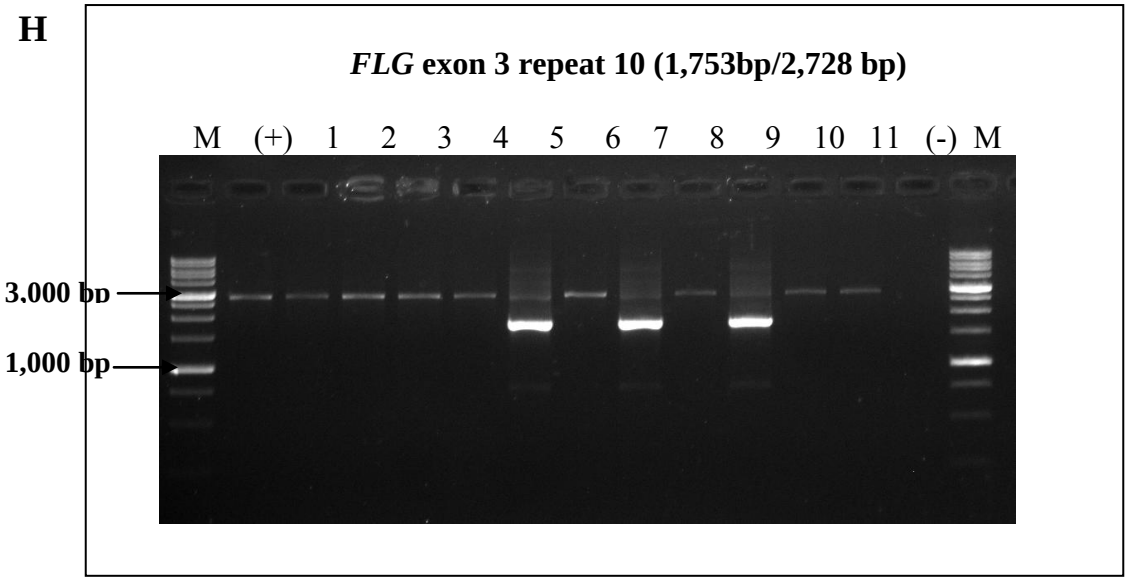
F


FLG exon 3 repeats 7-8 (1,224 bp)
M (-) (+) 1
3,000 bp
1,000 bp

G


FLG exon 3 repeats 9-10 (1,367 bp)
M (-) (+) 1 2
3,000 bp
1,000 bp

H


FLG exon 3 repeat 10 (1,753bp/2,728 bp)
M (+) 1 2 3 4 5 6 7 8 9 10 11 (-) M
3,000 bp
1,000 bp

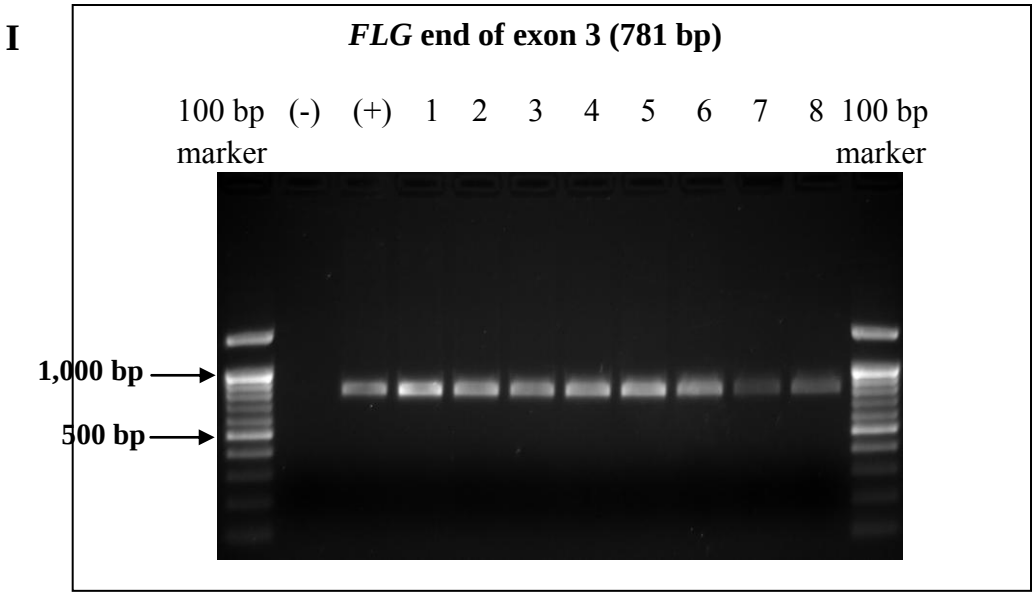


Figure 8. Gel electrophoresis of PCR products. (A) *FLG* exon 2, (B) exon 3 repeats 1-3, (C) repeats 3-5, (D) repeats 4-7, (E) repeats 7-10, (F) repeats 7-8, (G) repeats 9-10 , (H) repeat 10 and (I) the end of exon 3. M = 1 kb DNA marker; (-) = negative control; (+) = positive control from genomic DNA of normal control; Lanes 1-12 = patients with atopic dermatitis

Mutation analysis of *FLG*

PCR-sequencing of the entire coding sequence of *FLG* in 48 unrelated Thai individuals with atopic dermatitis identified four heterozygous mutations in *FLG*, c.1576G>T (p.G526X), c.4665delG (p.R1555SfsX1706), c.11944C>T (p.R3982X), and c.5717C>A (p.S1906X) (Figures 7-10) in 4 unrelated patients (8.3%). All of the mutations have never been previously described. Of the four novel mutations, three were nonsense mutations and one was a frameshift mutation, introducing a premature termination codon.

Patient 1: A girl with clinical features consistent with atopic dermatitis was found to have a nonsense mutation in repeat 1 of *FLG* exon 3. She was heterozygous for a G to T mutation at nucleotide position 1576 (c.1576G>T) (Figure 9). The mutation is expected to result in premature termination at codon 526 (p.G526X). Her clinical manifestations of atopic dermatitis began at 6 years of age. She had dry skin with palm and sole hyperlinearity. The eczematous lesions appeared on the flexures of arms and legs. She also had shrimp allergy and allergic rhinitis. Her total serum IgE (2040 KU/L) was higher than normal (144.50 KU/L in 5-13 year age group).

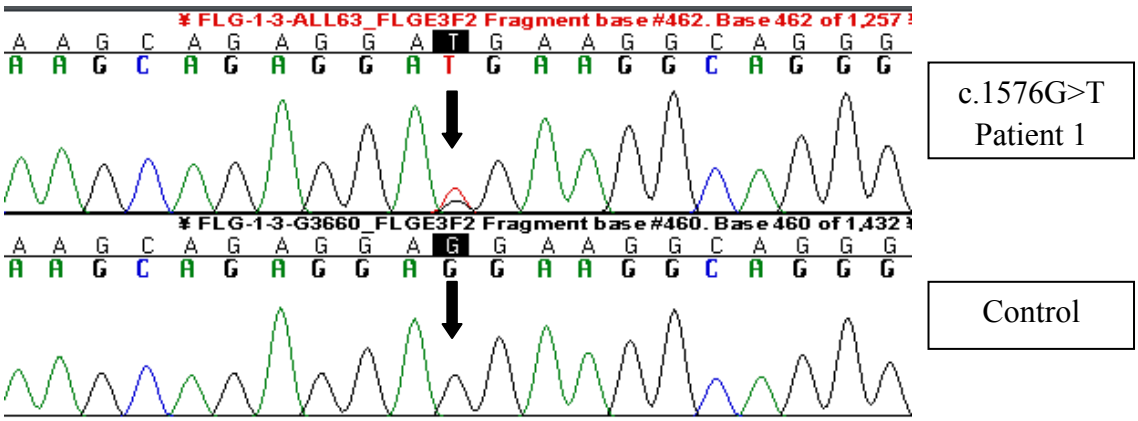


Figure 9. Chromatogram of patient 1. Upper panel shows a heterozygous mutation, c.1576G>T (p.G526X) in repeat 1 of *FLG* exon 3, resulting in a premature termination codon. Lower panel shows the normal sequence.

Patient 2: A girl with clinical features consistent with atopic dermatitis was found to have a frameshift mutation in repeat 4 of *FLG* exon 3. She was heterozygous for a single nucleotide deletion at nucleotide position 4665 (c.4665delG) (Figure 10). The frameshift mutation is expected to result in premature termination (p.R1555SfsX1706). Her clinical manifestations of atopic dermatitis began at 3 years of age. She had dry skin with palm and sole hyperlinearity. The eczematous lesions appeared on the flexure of arms and legs. She also had egg white allergy, allergic rhinitis, and allergic conjunctivitis. The total serum IgE was 103 KU/L that was higher than normal (86.97 KU/L in 2-5 year age group).

Patient 3: A boy with clinical features consistent with atopic dermatitis was found to have a frameshift mutation in repeat 4 of *FLG* exon 3. He was heterozygous for a G deletion at nucleotide position 4665 (c.4665delG) (Figure 10). He was the patient 2's older brother. His clinical manifestations of atopic dermatitis began at 2 years of age. He also had dry skin with palm and sole hyperlinearity. His eczematous lesions appeared on the flexures of arms and legs. He was allergic to shrimp. The total serum IgE (2060 KU/L) was higher than normal (86.97 KU/L in 2-5 year age group).

The father of patients 2 and 3: The father of patients 2 and 3 was found to harbor the similar mutation. He was heterozygous for a G deletion at nucleotide position 4665 (c.4665delG) as shown in figure 10. He had clinical features of atopic dermatitis when he was young. It went away when he got older.

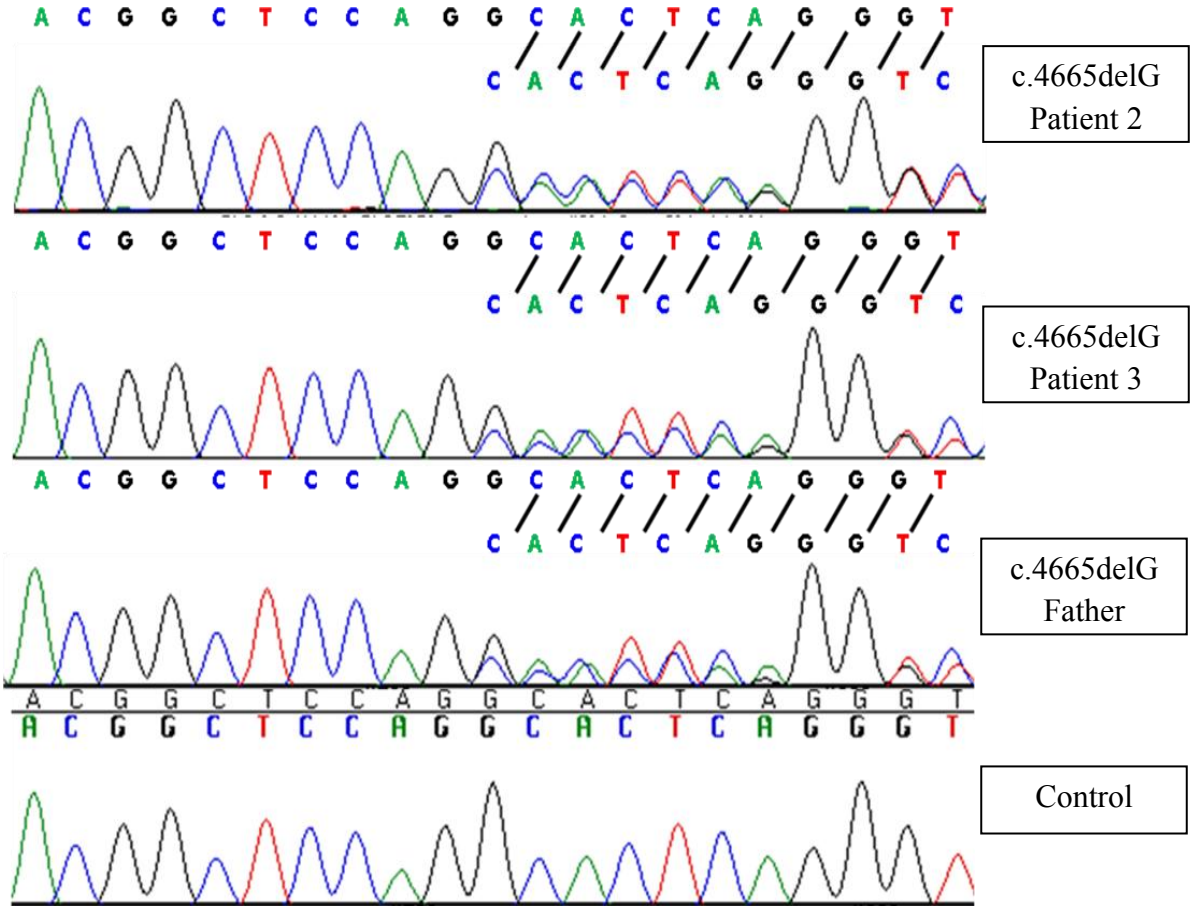


Figure 10. Chromatogram of patients 2, 3, and their father showing the heterozygous mutation, c.4665delG (p.R1555SfsX1706) in repeat 4 of *FLG* exon 3.

Patient 4: A girl with clinical features of atopic dermatitis was found to have a nonsense mutation in repeat 10.2 (duplication of repeat 10) of *FLG* exon 3. She was heterozygous for a C to T mutation at nucleotide position 11944 (c.11944C>T) as shown in figure 11. The mutation is expected to result in a premature termination at codon 3982 (p.R3982X). Her clinical manifestations of atopic dermatitis began at 3 years of age. She had dry skin and eczematous lesions on the flexural surfaces of arms and legs. She also had allergic rhinitis, house dust mite allergy, and cow milk allergy. The total serum IgE (2690 KU/L) was higher than normal (144.50 KU/L in 5-13 year age group).

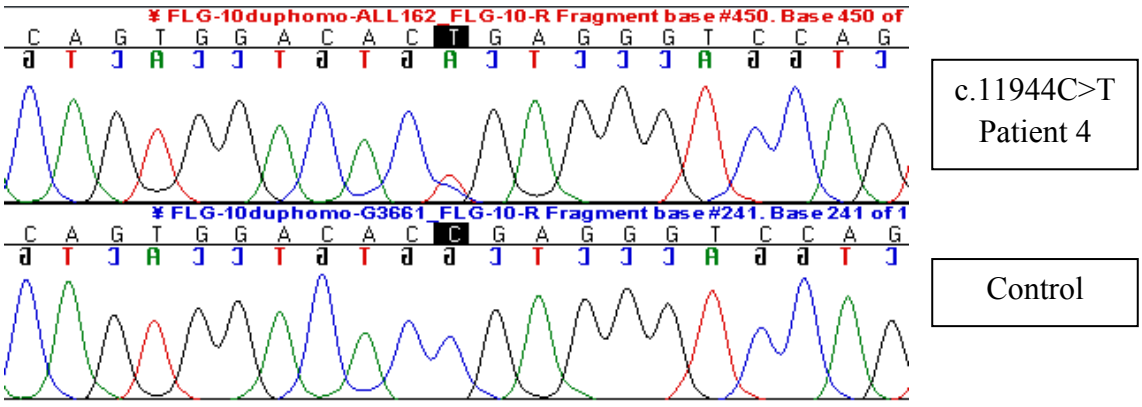


Figure 11. Chromatogram of patient 4. Upper panel shows a heterozygous mutation, c.11944C>T p. R3982X in repeat 10.2 (10 duplication) of *FLG* exon 3. Lower panel shows the normal sequence.

Patient 5: A woman with clinical features consistent with atopic dermatitis was found to have a nonsense mutation in repeat 5 of *FLG* exon 3. She was heterozygous for a T to G mutation at nucleotide position 5717 (c.5717C>A) (Figure 12). The mutation is expected to result in a premature termination at codon 1906 (p.S1906X).

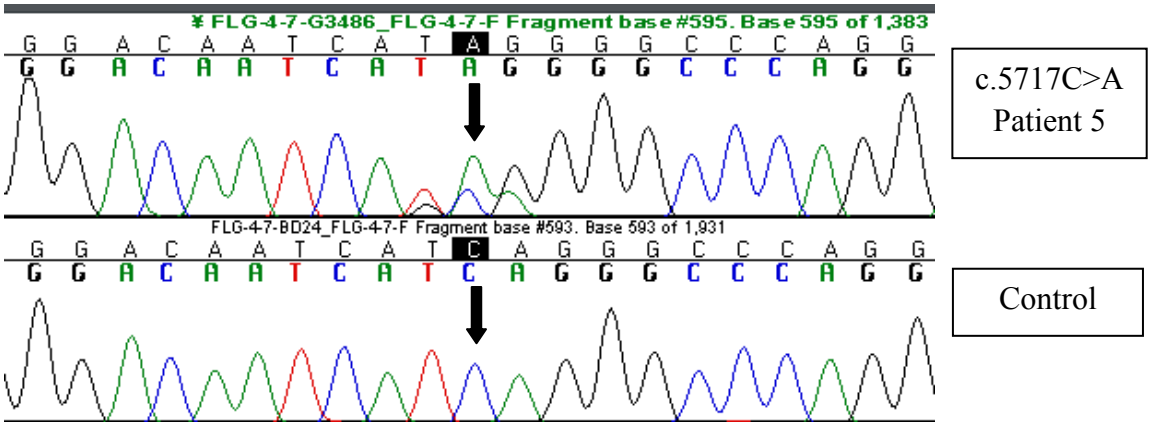


Figure 12. Chromatogram of patient 5. Upper panel shows a heterozygous mutation, c.5717C>A p.S1906X, in repeat 5 of *FLG* exon 3. Lower panel shows the normal sequence.

Confirmation and screening of four mutations in *FLG*

The mutations found in *FLG* were confirmed and screened in 100 ethnically-matched control chromosomes by PCR-direct sequencing or pyrosequencing of a short PCR fragment. PCR and direct sequencing of the patient and 50 unaffected controls was carried out to confirm and screen for the presence of the c.1576G>T (p.G526X), c.11944C>T (p.R3982X), and c.5717C>A (p.S1906X) mutations.

Pyrosequencing of a short PCR fragment comprising the *FLG* c.4665delG mutation was carried out to confirm and screen for the presence of the c.4665delG mutation in 50 unaffected controls. The result was shown in figure 13.

The results revealed that none of the four mutations were found in the controls.

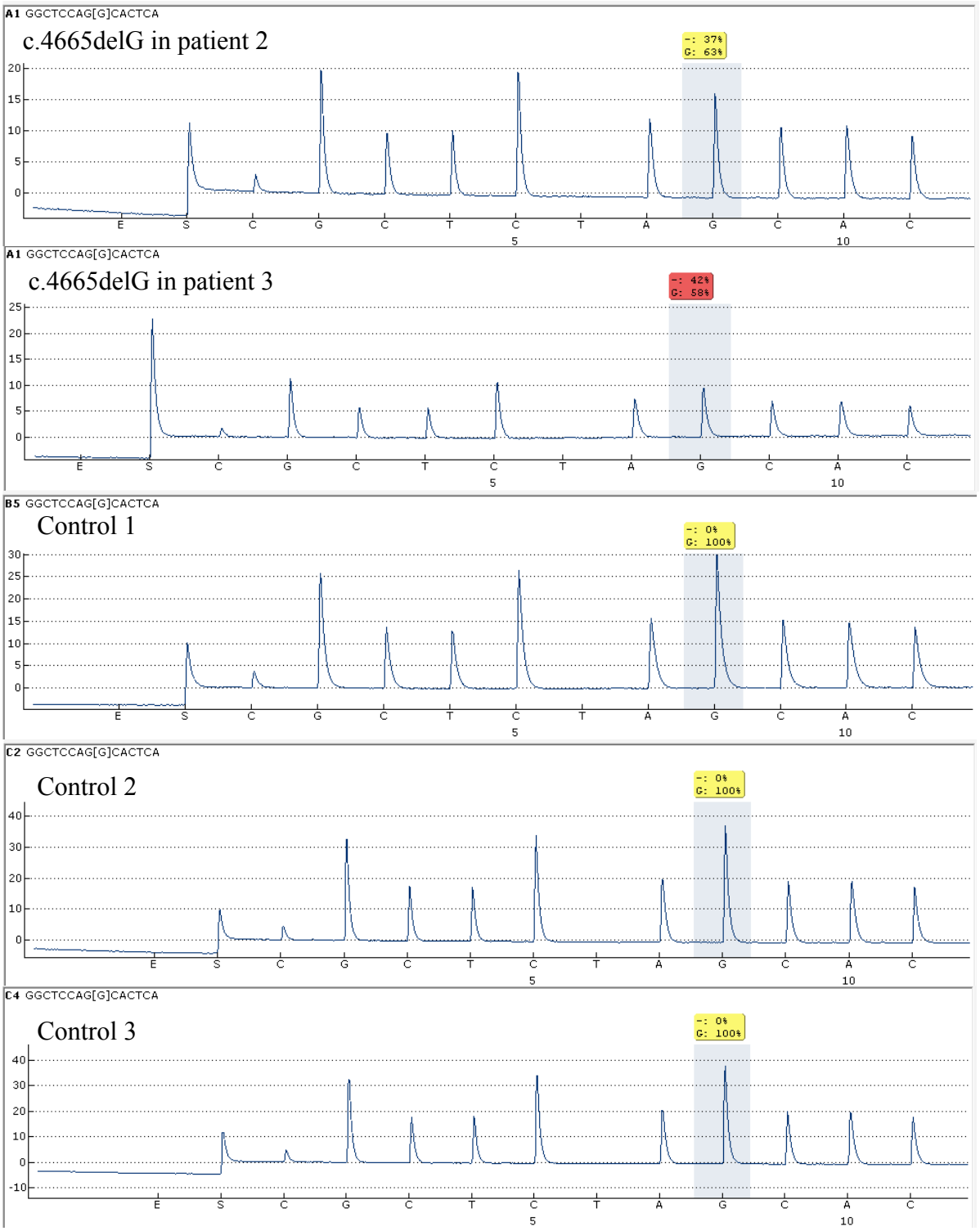


Figure 13. Pyrogram shows the presence of c.4665delG in patients 2 and 3 and absence of c.4665delG in 50 unaffected controls.

Table 13. *FLG* mutations in Thai patients with atopic dermatitis**.**

Mutation	protein	Repeat	Number of patient
c.1576G>T	p.G526X	1	1
c.4665delG	p.R1555SfsX1706	4	1 (both siblings)
c.5717C>A	p.S1906X	5	1
c.11944C>T	p.R3982X	10.2	1

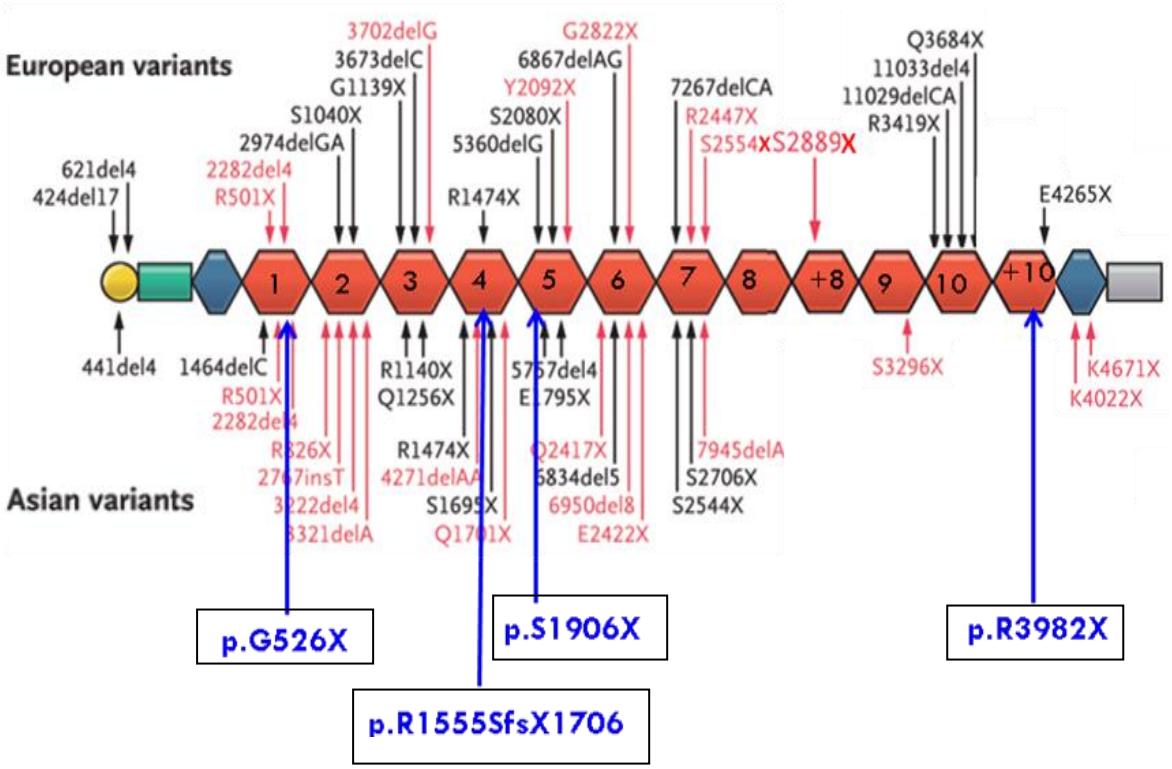


Figure 14. Mutations spectrum of *FLG* composed of novel *FLG* mutations in Thai patients with atopic dermatitis (p.G526X, p.R1555SfsX1706, p.R3982X, and p.S1906X).[58] Box indicates the novel *FLG* mutations in Thai patients with atopic dermatitis.

Mutation analysis of *SLC25A46*

For PCR-sequencing of the entire coding sequence of *SLC25A46* in 48 unrelated Thai individuals with atopic dermatitis, we found five single nucleotide polymorphisms (SNPs) as shown in table 14.

Table 14. SNPs in *SLC25A46* in atopic dermatitis patients

SNP	Nucleotide Change	Type	N
rs17446534	T>C	Synonymous variant	2
rs7736017	T>G	Intron variant	1
rs3213937	A>G	Intron variant	11
rs3214016	A>G	Intron variant	2
rs7724788	G>A	Synonymous variant	9

Mutation analysis of *TMEM232*

PCR-sequencing of the entire coding sequence of *TMEM232* in 48 unrelated Thai individuals with atopic dermatitis found nine SNPs as shown in table 15.

Table 15. SNPs in *TMEM232* in atopic dermatitis patients

SNP	Nucleotide Change	Type	N
rs17132237	T>A	Intron variant	1
rs1422495	A>C	Synonymous variant	6
rs4544868	A>G	Intron variant	15
rs6886702	T>G	Intron variant	28
rs61730920	T>C	Synonymous variant	1
rs72771461	A>G	Splice variant	1
rs139336689	A>G	Intron variant	1
rs6897672	C>A	Intron variant	1
rs148981452 insertion	insT	Intron variant	4

CHAPTER V
DISCUSSION AND CONCLUSION

FLG becomes the most significant genetic risk factor for atopic dermatitis after loss-of-function mutations in *FLG* have been identified as its major predisposing factor in 2007.[19] *FLG* not only shows a strong association with atopic dermatitis but also plays an essential role in the terminal differentiation of the epidermis and formation of skin barrier. *FLG* is located on chromosome 1q21. It contains three exons. The exon 3 is large (~12.7-14.7 kb) and encodes 10–12 filaggrin repeats that are varied in size within normal population.[9-10] The repeats have almost 100% similar at the DNA level. Therefore PCR-sequencing analysis of *FLG* is quite difficult and challenging. After sequencing of *FLG* in Thai patients with atopic dermatitis and normal controls was performed, we, for the first time, reported a *FLG* mutation spectrum in Thai patients with atopic dermatitis. We found four novel heterozygous mutations, c.1576G>T (p.G526X), c.4665delG (p.R1555SfsX1706), c.5717C>A (p.S1906X), and c.11944C>T (p.R3982X), which located in filaggrin repeat 1, 4, 5, and 10.2 (duplication of repeat 10), respectively.

FLG encodes a large insoluble profilaggrin, which is the main constituent of keratohyalin granules in the granular layer. When terminal differentiation of epidermis begins, the profilaggrin protein is proteolytically cleaved into multiple filaggrin peptides. Then they bind to keratin filaments for compaction and formation of keratinocytes at the granular layer-stratum corneum transitional zone. Subsequently, within the stratum corneum, filaggrin is degraded into amino acids and derivatives such as urocanic acid and pyrrolidone carboxylic acid. Both are natural moisturising factors, which contribute to moisture retention and UV protection. [23] Previous studies using immunohistological and Western blot analysis showed that the amount of profilaggrin was reduced and the processed filaggrins were absent in patients harboring *FLG* mutations. [19] It suggested that reduction or loss of filaggrin expression correlated with reduced natural moisturising factors and impaired epidermal barrier function contributing to excessively dry, flaky skin and defective skin barrier function in ichthyosis vulgaris and atopic dermatitis.

Even though no *in vitro* study has been performed to investigate the functional consequence of these mutations, there are several lines of evidence supporting them as pathogenic mutations. First, these mutations are nonsense and frameshift mutations introducing a premature truncated protein. Second, these mutations have not been reported to be a polymorphism in NCBI (http://www.ncbi.nlm.nih.gov/projects/SNP), Ensembl (http://www.ensembl.org/index.html) databases, 1000 Genomes, and dbSNP135. And lastly, they were not detected in 100 ethnically-matched control chromosomes.

The most common mutations in the European (R501X and 2282del4) and Asian (3321delA) including Chinese, Singaporean, Japanese, Korean and Taiwanese [18, 22] were not found in the Thai population. Our findings suggest that the Thai population may have a specific mutation in Thai patients with atopic dermatitis. As mentioned above, the *FLG* mutations are specific to each population. Therefore mutation screening in the Thai population cannot use the data obtained from the *FLG* mutations studies reported in other populations. Obtaining the common *FLG* mutations in each population is therefore necessary.

FLG mutations were present in 4 out of 48 unrelated Thai individuals with atopic dermatitis cases (8.3%). Moreover, no mutations were found in unaffected controls suggesting the importance of *FLG* mutations in our patients with atopic dermatitis. However, the frequency of our *FLG* mutations (10.2%) is lower than that in the Han Chinese (31.4%, n=261) (21), Japanese (27%, n=137) (35), Singapore (20.2%, n=425) and European populations (46%, n=228) (22). It might result from the difference of the environment. The environment in Europe and Thailand is clearly different. The weather in Thailand is hot and humid which does not support the development of atopic dermatitis. Trans-epidermal water loss is also reduced. In the other hand, the weather in Europe, China, Japan are mostly cold and dry. Such environment is good for development of atopic dermatitis. As for Singapore, the frequency was also higher. The different results may result from the difference of sample size and mix-populations in Singapore.

No mutations were identified in *SLC25A46* and *TMEM232*. However, we found SNPs in both genes. Our findings did not support the role of both genes in atopic dermatitis.

In summary, we have established a *FLG* sequencing protocol using PCR-direct sequencing for mutation identification. Using these methods, we identified four novel mutations, c.1576G>T (p.G526X), c.4665delG (p.R1555SfsX1706), c.5717C>A (p.S1906X), and c.11944C>T (p.R3982X) in Thai individuals with atopic dermatitis, expanding the mutational spectrum of *FLG*.

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APPENDICES

APPENDIX A
BUFFERS AND REAGENTS

1. 10X Tris borate buffer (10X TBE buffer)

Tris – base	100 g
Boric acid 55 g	0.5 M
EDTA (pH 8.0)	40 ml

Adjust volume to 1,000 ml with distilled water.

The solution was mixed and stored at room temperature.

2. 1X loading dye

Bromphenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	50 ml
1M Tris (pH 8.0)	1 ml

Distilled water until 100 ml Mix and store at 4°C

3. 1% Agarose gel (w/v)

Agarose	1.0 g
1X TBE	100 ml

Dissolve by heating in microwave oven and occasionally mix until no granules of agarose are visible.

4. Ethidium bromide

Ethidium bromide	10 mg
Distilled water	1 ml

Mix the solution and store at 4°C

5. 100 bp ladder

100 bp ladder stock	30 µl
TBE buffer	30 µl
1X loading dye	30 µl

Mix the solution and store at 4°C

6. 1k bp ladder

1k bp ladder stock	30 µl
TBE buffer	30 µl
1X loading dye	30 µl

Mix the solution and store at 4°C

APPENDIX B

CRITERIA FOR SELECTION OF CONTROLS

In this study, bloods from healthy controls were obtained from blood bank of Thai Red Cross. We used questionnaire to select unaffected ethnically-matched without atopic dermatitis, ichthyosis vulgaris, psoriasis, atopic disorders, and family history of atopic dermatitis, ichthyosis vulgaris and atopic disorders.

The questionnaires for selection were as follow:

Code number.....

วัน เดือน ปี ที่เก็บแบบสอบถาม .....

ชื่อ..... .....นามสกุล.....

ที่อยู่..... .....

.....

เบอร์โทรศัพท์..... .....

วัน เดือน ปี เกิด..... .....อายุ.....

เชื้อชาติ ปู่.....ย่า..... ตา..... ยาย..... ...

ท่านมีความพิการแต่กำเนิดใดๆหรือไม่ ไม่มี..... มี คือ.....

สมาชิกคนใดในครอบครัวของท่าน (ปู่ ย่า ตา ยาย , ลุง ป้า นา อา, พ่อ แม่ พี่ น้อง และบุตร) มีความพิการ

แต่กำเนิดหรือโรคประจำตัวใดๆหรือไม่ ไม่มี..... มี ได้แก่..... ....

ใครเป็น..... .....

โรคประจำตัวของท่าน.....

BIOGRAPHY

Ms. Piyamai Changate was born in Bangkok, the capital city of Thailand, in March 26th, 1989. In 2011, I received my Bachelor Degree of Science (2nd Class Honors) in Biology from Faculty of Science, Chulalongkorn University. I'm interested in Human and Molecular Genetics. So I had made a decision to study in Master Degree of Medical Science in Faculty of Medicine. During Master degree studies, I got Chulalongkorn university graduate scholarship to commemorate the 72nd anniversary of His Majesty King Bhumibol Adulyadej.