Original Article

Association of Filaggrin (*FLG*) Gene Polymorphism with Canine Atopic Dermatitis in Small Breed Dogs

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Abstract

As a cornified envelope protein, filaggrin (FLG) is involved in the formation and support of the skin barrier. *FLG* single nucleotide polymorphism (SNP) at the position 64,297,022 (rs22588227) on canine chromosome 17 has initially been described as being associated with canine atopic dermatitis (CAD) in Labrador Retrievers in UK. In this study, we have examined whether the mentioned FLG SNP is associated with a susceptibility to CAD in small breed dogs, comprising 21 Poodles, 17 Shih tzus and 3 Pugs. Twelve of these subjects were dogs with atopy and were assigned to the experiment group and the remaining 39 samples were healthy controls. The results showed no difference of the allele frequencies at the above-mentioned position between dogs with atopic dermatitis and the controls. However, this study assessed a naturally observed sequence diversity of FLG in the dog, identifying 13 new SNPs within canine FLG and a novel repeated sequence of FLG which had not appeared in dog genome databases. Allele frequencies demonstrated that 2 of the 13 novel observed polymorphisms at the locations 64,297,000 (p = 0.041, odds ratio = 3.920) and 64,297,126 (p = 0.043, odds ratio = 3.706) were plausibly associated with a susceptibility to CAD. The effect of all SNPs was dependent on one another with a strong linkage disequilibrium (D'≥0.89) in one haplotype block (frequencies ≥2%). This study suggests a role of FLG polymorphisms in CAD and also demonstrates the successful attempts to identify another unique fragment of the repeated FLG sequence and the novel SNPs. However, since small population was included in this study, the study should be repeated with a larger population.

Keywords: canine atopic dermatitis, dog, filaggrin, SNP

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บทคัดย่อ

ความสัมพันธ์ของความหลากหลายทางพันธุกรรมของยีนฟิแลกกรินกับโรคผิวหนังชนิดอะโทปีในสุนัข

กรรณาภรณ์ สุริยผล [1] ประพัฒน์ สุริยผล [2] มีนา สาริกะภูติ [1] ศิรินทร์ ธีระวัฒนศิริกุล [3] อัจฉริยา ไศละสูต [3]*

ฟิแลกกรินเป็นโปรตีนในกลุ่มคอร์นิไฟด์เอนเวลโลปซึ่งมีความสำคัญในการสร้างและค้ำจุนโครงสร้างผิวหนังชั้นนอก ก่อนหน้านี้มีรายงานการพบความสัมพันธ์ของสนิป (single nucleotide polymorphism, SNP) หรือความหลากหลายทางพันธุกรรมจากความแตกต่างของลำดับนิวคลีโอไทด์เบสเพียง 1 ตำแหน่งของยีนฟิแลกกรินที่ตำแหน่ง 64,297,022 (อาร์เอส 22588227) บนโครโมโซมที่ 17 ของสุนัขกับโรคผิวหนังชนิดอะโทปีในสุนัขพันธุ์ลาบราดอร์รีทริฟเวอร์ในสหราชอาณาจักร ในการศึกษาครั้งนี้เราได้ศึกษา ความสัมพันธ์ของตำแหน่งสนิปดังกล่าวกับการเกิดโรคผิวหนังชนิดอะโทปีในสุนัขพันธุ์เล็กจำนวน 41 ตัว ประกอบด้วย พันธุ์พุดเดิ้ล 21 ตัว พันธุ์ชิสุห์ 3 ตัว พันธุ์ปั๊ก 3 ตัว โดยมีสุนัข 12 ตัวซึ่งป่วยเป็นโรคผิวหนังชนิดอะโทปีเป็นกลุ่มทดลองและสุนัข 39 ตัวซึ่งไม่ป่วยเป็นกลุ่มควบคุม จากการศึกษาไม่พบความแตกต่างของสนิปที่ตำแหน่งดังกล่าวในสุนัขที่ป่วยเป็นโรคอะโทปีและสุนัขปกติ แต่พบสนิปใหม่ 13 ตำแหน่งในยีนฟิแลกกรินและพบท่อนซ้ำท่อนใหม่ของยีนซึ่งไม่ปรากฏในฐานข้อมูลจีโนมของสุนัข จากความถี่อัลลีลแสดงว่าสนิปที่ตำแหน่ง 64,297,000 (p=0.041, อัตราส่วนออด = 3.920) และ 64,297,126 (p=0.043, อัตราส่วนออด = 3.706) อาจมีความสัมพันธ์กับความไวในการเกิดโรคอะโทปี สนิปทั้ง 13 ตำแหน่งมีลิงค์เกจดิสอีควิลิเบรียม (linkage disequilibrium; LD) ต่อกันและกันที่แข็งแรงมาก (D'≥0.89) ซึ่งแสดงถึงการพบสนิปในประชากรบ่อยกว่าที่จะพบโดยบังเอิญ นอกจากนี้ยังพบรูปแบบการกระจายของสนิปหลายๆตำแหน่งอยู่ใกล้กันบนโครโมโซมเดียวกันเป็นแฮปโลไทป์บล็อก (haplotype block) ซึ่งแสดงว่าสนิปอัลลีลที่อยู่ใกล้กันมีแนวโน้มที่จะถูกถ่ายทอดไปด้วยกันทั้งหมดเป็นชุดโดยไม่มีการเปลี่ยนแปลง จำนวน 1 บล็อกด้วยความถี่มากกว่าหรือเท่ากับร้อยละ 2 การศึกษาครั้งนี้นำเสนอบทบาทของความหลากหลายทางพันธุกรรมของยีนฟิแลกกรินต่อโรคผิวหนังชนิดอะโทปีและแสดงถึงการหาท่อนของยีนฟิแลกกรินท่อนใหม่และการพบสนิปใหม่ของยีนดังกล่าว อย่างไรก็ดีเนื่องจากจำนวนสุนัขที่ทำการศึกษามีจำนวนไม่มาก จึงควรทำการศึกษาซ้ำในกลุ่มประชากรที่ใหญ่ขึ้นต่อไป

คำสำคัญ: โรคผิวหนังชนิดอะโทปีในสุนัข สุนัข ฟิแลกกริน สนิป

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Introduction

Canine atopic dermatitis (CAD) is defined as "a genetically-predisposed inflammatory and pruritic skin disease with characteristic clinical features that is associated with IgE antibodies, most commonly directed against environmental allergens" (Halliwell, 2006). CAD naturally shares several characteristics with human AD (HAD), including familiar predisposition, histopathological findings characterized by spongiotic dermatitis, with the accumulation of mononuclear, epidermal and dermal IgEþCD1cþ cells and epidermal eosinophils, clinical presentation with pruritic domination and immunologic aspects associated with IgE antibodies under environmental influence. Hence, dog is considered to be a model of HAD (Rhodes et al., 1987; Olivry et al., 1988; Willemse, 1988; Hill et al., 2001; Marsella and Girolomoni, 2009).

There is increasing evidence that several gene mutations are associated with HAD. Some gene polymorphisms are relevant to impaired immune function such as interferon-gamma (IFNγ) (Hussein et al., 2009), interleukin (IL)-13 receptor alpha 2 (Hussein et al., 2011), toll-like receptor 2 (Mrabet-Dahbi et al., 2008; Oh et al., 2009; Roduit et al., 2011), IL-12 alpha and IL-12 receptor beta 1 (Namkung et al., 2010a), defensin beta 1 (Kim et al., 2009) and interferon regulatory factor 2 (Nishio et al., 2001). Other mutations are associated with skin barrier formation, including serine protease inhibitor, kazal type 5 (SPINK 5) (Kato et al., 2003; Nishio et al., 2003; Kabesch et al. 2004; Liu et al., 2009; Namkung et al., 2010b), kallikrein-related peptidase 7 or stratum corneum chymotryptic enzyme (KLK 7/SCCE)

(Vasilopoulos et al., 2011) and filaggrin (FLG) (Palmer et al., 2006; Ekelund et al., 2008; Kang et al., 2009; Nemoto-Hasebe et al., 2009; Greisenegger et al., 2010; Chen et al., 2011; Zhang et al., 2011). The combination of both types of gene mutation was observed i.e. between FLG and IL-10, IL13 (Lesiak et al., 2011). In addition, based on comparison with human AD and previous studies on CAD, the CAD is assumed to be a polygenic or oligogenic inheritance influenced by gene-environment interactions. A number of gene mutations have been reported to be associated with CAD but only in some breeds and geographical regions such as MS4A2 and INPPL1 in Shiba dogs in Japan and DPP4 and FLG in Labradors in the UK (Wood et al., 2010).

FLG is a structural protein necessary for skin barrier formation. It is a component of epidermis cornified cell envelope in human (Simon et al., 1996). FLG causes cells to get compact into flattened squamous by aggregating the keratin filaments (Rawlings et al., 1994; Rawlings and Matts, 2005). In human AD, filaggrin plays a crucial role (Weidinger et al., 2006; Morar et al., 2007; McGrath, 2008). Loss-of-function mutations in the FLG gene have been reported to be associated with human AD for 14-56% (Palmer et al., 2006; Smith et al., 2006; Weidinger et al., 2006; Irvine 2007; Sandilands et al., 2007). In CAD, single nucleotide polymorphism (SNP) within FLG located on chromosome 17 at position 64,297,022 (rs22588227) has recently been shown to be significantly associated with CAD in Labrador Retrievers in the UK (Wood et al., 2010).

In this study, we have further investigated whether the previously published FLG mutation is associated with CAD in small breed dogs, namely Poodles, Shih tzus and Pugs.

Materials and Methods

Sample collection: Forty one dog subjects were recruited from the Small Animal Hospital at the Faculty of Veterinary Science, Chulalongkorn University and private small animal clinics. Dogs from small breeds were divided into 2 groups. Group 1 was dogs with AD, comprising 8 Poodles, 2 Shih tzus and 2 Pugs, and group 2 was healthy controls, comprising 13 Poodles, 15 Shih tzus and 1 Pug. The diagnosis of CAD was based on compatible history and clinical signs, the exclusion of other causes of pruritus and 5 signs or more under Favrot's 2010 criteria (Favrot et al, 2010; Olivry et al., 2010). Bacterial and yeast infections and ectoparasite infestation were controlled prior to inclusion. No anti-inflammatory medication was given for at least 3 weeks prior to examination. Control samples were from clinically normal dogs. Blood samples were collected into EDTA-coated tubes, aliquot and kept at -20^{0}C. Genomic DNA was isolated from 200 µL blood, using a DNA isolation kit according to the manufacturer's instructions (the HiYield Genomic DNA Extraction kit, RBC Bioscience, Taipei, Taiwan). The amount of DNA extracted was quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The isolated DNA was stored at -20^{0}C. The study was conducted under Animal Use Protocol number 1031036, authorized by the Chulalongkorn University Animal Care and Use Committee (CU-ACUC).

Analysis of FLG polymorphisms: Genotyping was carried out by DNA sequencing method, franking the reported FLG mutation position. Primer setting was based on the published DNA sequence for canine FLG (ENSCAFG00000023034). The primers for the candidate region were designed using Primer3 (version 0.4.0) free software (Rozen and Skaletsky, 2000) and were checked for specificity using the UCSC In-Silico PCR program (http://genome.ucsc.edu). A primer walking from both 5′ and 3′ terminals of the amplified product was performed, using 2 pairs of primer sets to obtain a specific polymerase chain reaction (PCR) product. The outer primers amplified a 2,260 bp product. The fwd inner-1 and rev inner primer set was designed to overlap the amplified product by the outer primers in order to sequence it. In addition, the fwd inner-2 primer was utilized to confirmed SNP sequences obtained from the rev outer primer. Table 1 summarizes primers and the lengths of PCR products. All reactions were carried out in the presence of 300 ng of template DNA in 50 µl reaction mixtures, containing 0.2 µM of each primer, 0.2 mM of each dNTP, 2 mM $MgSO_4$, and 2 units Platinum Taq DNA Polymerase High Fidelity (Life Technologies; Carlsbad, CA). Reactions were performed in GeneAmp PCR System 9700 thermocycler (Life Technologies; Carlsbad, CA), according to the following thermocycling conditions: one cycle of 94^{o}C for 2 min; 40 cycles of 94^{0}C for 15 sec, 53^{0}C for 30 sec and 68^{0}C for 3 min; and one cycle of 68^{0}C for 5 min. After the amplification reaction, the samples were held at -20^{0}C until analysis. The PCR products were separated by 1% agarose gel electrophoresis. The samples were gel-purified by HiYield Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience; Taipei, Taiwan) according to the manufacturer's instructions and were submitted for automated DNA sequencing to verify the SNP identity of the amplified PCR-fragments.

Table 1 Primer sequences and their locations

	Sequences (5′ –> 3′)	Locations	Lengths of PCR products (bp)
Fwd outer P′	CCTTTGTCAGGATATTTCAGCA	64,294,986	2,389
Rev outer P′	TTAACGACCCGTGGAGATTC	64,297,374	
Fwd inner-1 P′	TGTGTGGGTTCATATTCCTACAA	64,295,788	
Rev inner P′	GTCCAGGTCCAGTCAGAGGA	64,296,667	
Fwd inner-2 P′	TGTCTGTCTACGGTGCTGAGA	64,296,827	

Sequences were aligned to the draft Ensembl and NCBI dog genome databases (Canis_familiaris LATESTGP and dog build 2 genome databases, respectively) (Flicek et al., 2011; Gertz, 2011), based on the whole genome shotgun (WGS) assembly CanFam version 2.0 (Lindblad-Toh et al., 2005), using a BLASTN (the Nucleotide-nucleotide Basic Local Alignment Search Tool) 2.2.24+ Program (Zhang et al., 2000). The fragments were also aligned to the UCSC dog genome draft (Fujita et al., 2011), using a BLAT (the BLAST-like Alignment Tool) Program client/server version (Kent, 2002) and compared to the Broad SNP set (Lindblad-Toh et al., 2005).

Statistical analysis: The Hardy-Weinberg equilibrium (HWE), which shows the equilibrium of allele and genotype frequencies in a population from generation to generation, was tested for each SNP in cases and controls. The significant deviation from the HWE suggests that a mistake may occur for that SNP and require a double check in the genotyping. Differences in the allele frequencies between cases and controls were assessed with the Fisher's exact test. The Haploview 3.32 program was used to compute pairwise linkage disequilibrium (LD) statistics, Haplotype block and frequencies (Barrett et al., 2005). Standardized disequilibrium D' and r2 were plotted. The odds ratio (OR) and relative risk (RR) were used to demonstrate the association of SNPs in the canine genome draft (CGD), version May 2005 and alternative alleles to CAD. The cumulative effect of SNP genotypes was identified as susceptible (OR >1) or protective (OR <1) between the CAD cases and the controls. All P-values were derived from a two-sided test and *p*-values <0.05 were considered statistically significant.

Results

FLG re-sequencing: The exons 5-7 of the FLG encode all 4 predicted filaggrin repeats that are similar at the DNA level. This repetitive DNA renders the region of interest difficult for PCR and sequence analysis. PCR with the outer primer set, reported in this study, provided a single band although the product size was larger than expected. From FLG SNP reported by Wood et al. (2010), we re-sequenced the product, using our primers. Local similarity between obtained sequences and the CGD was searched using BLAST (NCBI, Ensembl) and BLAT (UCSC) programs. Both programs by NCBI and Ensembl provided similar results in that the fragment was on chromosome 17 of the canine genome draft sequence at locations 64,295,028 to 64,297,315 (2,288 bp). We manually realigned the sequences obtained from the rev outer primer to the CGD and we observed a 126 bp gap on chromosome 17 that did not appear in the dog genome. The gap started at location 64,297,250 followed by location 64,297,125 to 64,297,249 on CGD (Fig 1). It is likely to be homologous to several repeats on predicted FLG at the chromosome such as locations 64,301,940-64,302,064, 64,301,814- 64,301,938 and 64,300,419-64,300,543. Without the gap, the sequence was a 100% match to the CGD.

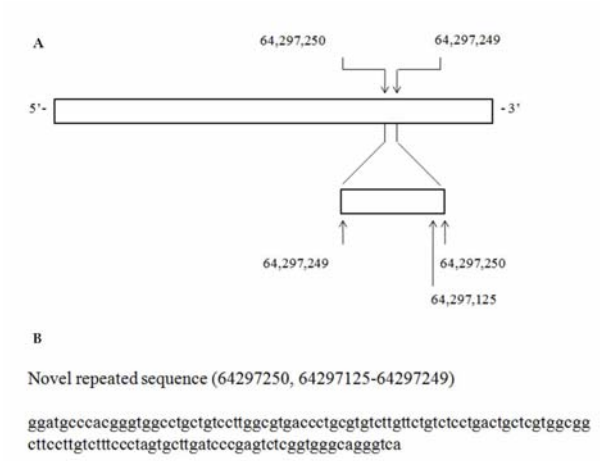

Figure 1 Novel repeated sequence of *FLG*. The sequence on chromosome 17 of the CGD, version May 2005, which was a minus strand of FLG was represented (A). Nucleotide sequence of a new repeated sequence is demonstrated (B).

Similar sequencing results in all provided samples ensured a unique amplified FLG fragment. However, the inserted nucleotides did not affect the reading frame of the FLG.

Genetic variation: From the consensus sequencing of the amplicons by a rev outer primer, thirteen SNPs in the exon 7 of FLG were genotyped. All SNPs were in HWE among both cases and controls (data not shown). A fragment of the analyzed sequence at location 64,296,737 to 64,297,315 harbored 13 SNPs which had not previously been reported on the SNP database (dbSNP) (Lindblad-Toh et al., 2005) regardless of whether they had the CAD or not (Table 2). The allele frequencies of 13 SNPs of FLG in dogs with atopic dermatitis and controls are presented in Table 2. Four of them are in the new repeated sequence. Allele frequencies demonstrated that 2 of the novel 13 observed polymorphisms (64,297,000 and 64,297,126) were likely to be significantly associated with susceptibility to CAD, using a significant *p*-value threshold of 0.05. The odds ratio and the relative risk indicate that both were susceptible SNPs (Table 2). However, no statistically significant association was observed for any of the genotype frequencies of SNPs between the CAD group and the control subjects ($p>0.05$) (data not shown).

Haplotype analysis: All 13 SNPs were located in 1 haplotype block with 7 haplotype patterns for ≥2% frequencies (Table 3). Two haplotype block patterns (GACCGCTTTTCGC and ACAGATGCGCGAT) were clearly dominated with frequencies 60.8% and 22.5% respectively, which suggests that the magnitude of LD between each SNP was high, with pair-wise D' values that were ≥0.89 (Fig 2). The distribution of the two most frequent haplotype patterns in dogs with AD and normal are shown in Table 4. The distribution suggested that the haplotype pattern 1 (GACCGCTTTTCGC) might be associated with CAD in Poodles, whereas haplotype pattern 2 (ACAGATGCGCGAT) might be associated with CAD in Shih tzus.

Table 2 Allele frequencies of single nucleotide polymorphisms (SNPs) in the filaggrin (FLG) gene of small breed dogs

Chromosomal position (bp)	Alleles		Alternative allele frequency, n(%)		*P* value	OR (CGD) (95%CI)	RR (CGD) (95%CI)	OR (Alt) (95%CI)	RR (Alt) (95%CI)
	CGD	Alt	CAD	Controls					
64,296,925	A	G	21(20.588)	58(56.000)	0.265	0.400 (0.116-1.405)	0.478 (0.157-1.288)	2.500 (0.712-8.641)	2.091 (0.777-6.366)
64,297,000	C	A	21(20.588)	50(49.020)	0.041**	0.255 (0.075-0.881)	0.327 (0.107-0.907)	3.920 (1.136-13.335)	3.056 (1.103-9.351)
64,297,126	A	C	21(20.588)	51(50.000)	0.043**	0.270 (0.079-0.933)	0.343 (0.112-0.948)	3.706 (1.072-12.622)	2.917 (1.055-8.921)
64,297,148	C	G	3(2.941)	24(23.529)	0.112	3.111 (0.895-10.645)	2.52 (0.919-7.697)	0.321 (0.094-1.117)	0.397 (0.130-1.088)
64,297,153	G	A	3(2.941)	26(25.490)	0.069	3.500 (1.011-11.937)	2.781 (1.008-8.502)	0.286 (0.084-0.989)	0.360 (0.118-0.992)
64,297,154	C	T	3(2.941)	25(24.510)	0.071	3.302 (0.952-11.279)	2.649 (0.963-8.094)	0.303 (0.089-1.05)	0.378 (0.124-1.038)
64,297,171	G	T	19(18.627)	47(46.078)	0.142	0.399 (0.140-1.147)	0.482 (0.195-1.110)	2.506 (0.872-7.137)	2.073 (0.901-5.120)
64,297,173	C	T	19(18.627)	49(48.039)	0.215	0.445 (0.156-1.281)	0.526 (0.213-1.207)	2.249 (0.780-6.418)	1.900 (0.829-4.690)
64,297,222	G	T	19(18.627)	49(48.039)	0.215	0.445 (0.156-1.281)	0.526 (0.213-1.207)	2.249 (0.780-6.418)	1.9000 (0.829-4.690)
64,297,142*	T	C	3(2.941)	20(19.608)	0.265	2.414 (0.688-8.335)	2.038 (0.756-6.207)	0.414 (0.12-1.454)	0.491 (0.161-1.322)
64,297,148*	C	G	3(2.941)	22(21.569)	0.175	2.750 (0.788-9.448)	2.273 (0.836-6.933)	0.364 (0.106-1.269)	0.440 (0.144-1.197)
64,297,153*	G	A	3(2.941)	22(21.569)	0.175	2.750 (0.788-9.448)	2.273 (0.836-6.933)	0.364 (0.106-1.269)	0.440 (0.144-1.197)
64,297,154*	C	T	3(2.941)	22(21.569)	0.175	2.750 (0.788-9.448)	2.273 (0.836-6.933)	0.364 (0.106-1.269)	0.440 (0.144-1.197)

Position of the respective markers is indicated by the genomic location on chromosome 17. In the table, CAD represents canine atopic dermatitis, CGD represents canine genome draft, version May 2005, Alt represents alternative nucleotides from the CGD, OR represents odds ratio, RR represents relative risk, CI represents confidence interval. P values were calculated by the Fisher's Exact Test using a 2×2 contingency table (vs control). An asterisk indicates SNP in repeat region. A double asterisk indicates significant difference (p <0.05)

Discussion

Since CAD is a complex disease and not all the factors contributing to CAD pathogenesis have been identified, the genes involved in skin barrier formation are probably implicated in the etiopathogenesis of CAD. Particular attention was given to the FLG SNP at location 64,297,022 (rs22588227) which had initially been indicated to be associated with CAD in 23 Labrador Retrievers with atopy and 75 controls in the UK (Wood et al., 2010). The SNP site was in the FLG gene on chromosome 17 at position 64,297,022. However, in this study, both affected and non-affected animals presented no variation at that position, hence, the association of the reported SNP and CAD was excluded. A linkage analysis of canine FLG in West Highland White Terriers (WHWT) was previously performed, but no obvious correlation between FLG and AD was found (Barros Roque et al., 2009). From the neighbour-joining trees based on allele sharing of SNPs and sharing of 10-SNP haplotypes for individuals and breed/population groupings, Poodles (working dogs), Shih tzus and Pugs (toy dogs) had a close genetic relationship which was much closer than that with a Labrador Retriever (Retriever) or WHWT (small terrier) groups (Vonholdt et al., 2010). These data supports our decision on combining three small breeds in association analysis to increase the power of statistical analysis.

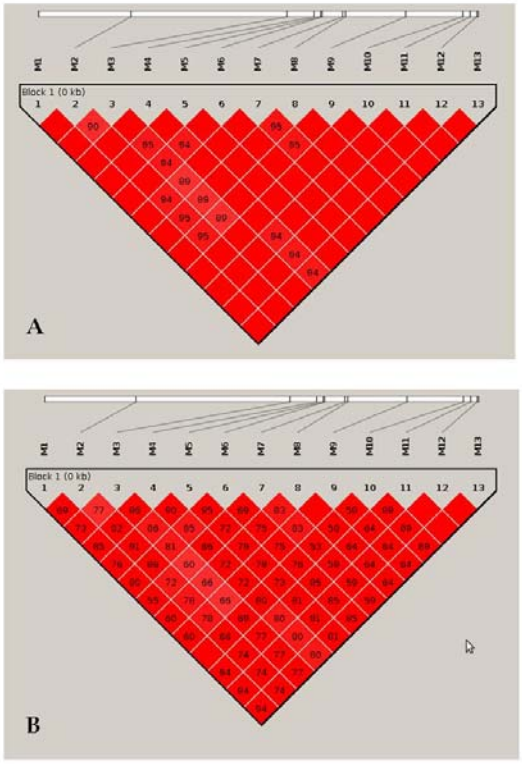

Figure 2 Structure of linkage disequilibrium (LD) plotted for 13 SNPs in the FLG gene. The D′ value (A) and r^2 value (B) that correspond to each SNP pair are expressed as a percentage and shown within the respective square. The 13 SNPs constitute a haplotype block that spans 356 bp of *FLG*.

Table 4 The percent frequencies of two haplotype blocks in dogs with AD and normals*

Haplotype block	Poodles		Shih tzus		Pugs	
	CAD	Control	CAD	Control	CAD	Control
1. GACCGCTTTTCGC	100.0	82.6	0.0	60.0	100.0	100.0
2. ACAGATGCGCGAT	0.0	39.0	100.0	40.0	50.0	0.0

*The percent frequencies were calculated from percent numbers of dogs with alleles (1 or 2) located in a haplotype block divided by the total number of dogs in each breed.

Table 3 Haplotype frequencies of *FLG* SNPs

Haplotypes	Frequency (%)
1. GACCGCTTTTCGC	60.8
2. ACAGATGCGCGAT	22.5
3. GACCGCGTTTCGC	3.0
4. GACCGCGCGTCGC	2.9
5. GAACGCTTTTCGC	2.0
6. GCCCGCGCGTCGC	2.0
7. GCAGATGCGTCGC	2.0

In order to search for new SNP mutations, we aligned DNA fragments of 2,288 bp to draft dog genome databases, using both BLAST (NBCI and Ensembl) and BLAT (UCSC) programs. The BLAST program is used to align a nucleotide sequence to a draft dog genome in NCBI and Ensembl which are believed to use the same draft dog genome database. The BLAT is a faster tool for finding alignments to DNA sequences with different structures from a BLAST program. The aligned results show the identical DNA fragments on the same chromosomal location. We found a novel repeated sequence of a 126 bp fragment at chromosome 17 which is likely to be homologous to a number of repeats on predicted FLG. The draft dog genome, Broad/canFam2.0, was obtained from whole-genome shotgun sequencing and assembly of 2.4 Gb. (http://genome.ucsc.edu /cgi-bin/hgGateway?db=canFam2) which required overlapping reads for each segment of the original DNA. Since FLG was predicted to contain a number of repetitive sequences, a putative error in sequence assembly could occur. Hence, more attention should be paid to SNP searching in any repeated sequence as canine FLG.

From the allele frequencies results, two SNPs are likely to be associated with CAD. Odds ratio and a relative risk of more than 1 together with the *p* values <0.05 demonstrated that the SNPs in alternative alleles at the genomic location 64297000 and 64297126 were plausibly associated with CAD. Our data support the role of FLG in CAD since the significant polymorphism was observed in FLG gene. In addition, from the analysis of 2 haplotype blocks with the two highest percent frequencies (GACCGCTTTTCGC, 60.8% and ACAGATGCGCGAT, 22.5%), we found that all 8 Poodles with atopy had the same pattern, GACCGCTTTTCGC, with 87.5% (7 in 8 dogs) homozygosity (Table 4). The close relationship between Poodles with CAD and the block GACCGCTTTTCGC should further be investigated. Since FLG nucleotide sequence in the canine genome draft is composed of several repeats which obstruct the identification of the unique fragment, this study demonstrated the successful attempts to identify another repeated FLG fragment together with novel SNPs. However, since a small number of dogs were included in this study, study of larger dog populations is suggested to demonstrate the actual relationship between FLG mutations and CAD.

In conclusion, we identified a novel repeated fragment and several new SNPs in the FLG gene of small breed dogs. No association was found between the previously reported SNP and the CAD, but we found association between two new SNPs in the FLG gene and the CAD. The data suggest a role of FLG polymorphisms in CAD. However, the importance of FLG in the development of canine atopic dermatitis should be confirmed in future studies with larger cohorts and the role of skin barrier function contributing to the pathogenesis of CAD needs to be further investigated.

Acknowledgements

We would like to express our thanks to Dr. Tossaporn Nakbed and Dr. Jakkaphan Wannawong for sample collections. This study was supported by the Thailand Research Fund and the Office of the Higher Education Commission (Grant number: MRG5180171, 2008-2010).

References

Barrett, J.C., Fry, B., Maller, J. and Daly, M.J. 2005. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 21: 263-265.

Barros Roque, J., O'Leary, C.A. and Kyaw-Tanner, M. 2009. Haplotype sharing excludes canine orthologous filaggrin locus in atopy in West Highland White Terriers. Anim Genet. 40: 793-794.

Chen, H., Common, J.E., Haines, R.L., Balakrishnan, A., Brown, S.J., Goh, C.S., Cordell, H.J., Sandilands, A., Campbell, L.E., Kroboth, K., Irvine, A.D., Goh, D.L., Tang, M.B., Van Bever, H.P., Giam, Y.C., McLean, W.H. and Lane, E.B. 2011. Wide spectrum of filaggrin-null mutations in atopic dermatitis highlights differences between Singaporean Chinese and European populations. Br J Dermatol. 165: 106-114.

Ekelund, E., Liedén, A., Link, J., Lee, S.P., D'Amato, M., Palmer, C.N., Kockum, I. and Bradley, M. 2008. Loss-of-function variants of the filaggrin gene are associated with atopic eczema and associated phenotypes in Swedish families. Acta Derm Venereol. 88: 15-19.

Favrot, C., Steffan, J., Seewald, W. and Picco, F. 2010.

A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. Vet Dermatol. 21: 23-31.

Flicek, P., Amode, M.R., Barrell, D., Beal, K., Brent, S., Chen, Y., Clapham, P., Coates, G., Fairley, S., Fitzgerald, S., Gordon, L., Hendrix, M., Hourlier, T., Johnson, N., Kähäri, A., Keefe, D., Keenan, S., Kinsella, R., Kokocinski, F., Kulesha, E., Larsson, P., Longden, I., McLaren, W., Overduin, B., Pritchard, B., Riat, H.S., Rios, D., Ritchie, G.R., Ruffier, M., Schuster, M., Sobral, D., Spudich, G., Tang, Y.A., Trevanion, S., Vandrovcova, J., Vilella, A.J., White, S., Wilder, S.P., Zadissa, A., Zamora, J., Aken, B.L., Birney, E., Cunningham, F., Dunham, I., Durbin, R., Fernández-Suarez, X.M., Herrero, J., Hubbard, T.J., Parker, A., Proctor, G., Vogel, J. and Searle, S.M. 2011. Ensembl 2011. Nucleic Acids Res. 39:D800–806.

Fujita, P.A., Rhead, B., Zweig, A.S., Hinrichs, A.S., Karolchik, D., Cline, M.S., Goldman, M., Barber, G.P., Clawson, H., Coelho, A., Diekhans, M., Dreszer, T.R., Giardine, B.M., Harte, R.A., Hillman-Jackson, J., Hsu, F., Kirkup, V., Kuhn, R.M., Learned, K., Li, C.H., Meyer, L.R., Pohl, A., Raney, B.J., Rosenbloom, K.R., Smith, K.E., Haussler, D. and Kent, W.J. 2011. The UCSC Genome Browser database: update 2011. Nucleic Acids Res. 39: D876-882.

Gertz, E.M. 2011. "BLAST Scoring Parameters." [Online]. Available: ftp://ftp.ncbi.nlm.nih.gov/blast/documents/developer/scoring.pdf. last edited on March 16, 2005.

Greisenegger, E., Novak, N., Maintz, L., Bieber, T., Zimprich, F., Haubenberger, D., Gleiss, A., Stingl, G., Kopp, T. and Zimprich, A. 2010. Analysis of four prevalent filaggrin mutations (R501X, 2282del4, R2447X and S3247X) in Austrian and German patients with atopic dermatitis. J Eur Acad Dermatol Venereol. 24: 607-610.

Halliwell, R. 2006. Revised nomenclature for veterinary allergy. Vet Immunol Immunopathol. 114: 2007–2008.

Hill, P.B., Hillier, A. and Olivry, T. 2001. The ACVD task force on canine atopic dermatitis (VI): IgE-induced immediate and late-phase reactions, two inflammatory sequences at sites of intradermal allergen injections. Vet Immunol Immunopathol. 81: 199-204.

Hussein, Y.M., Ahmad, A.S., Ibrahem, M.M., El Tarhouny, S.A., Shalaby, S.M., Elshal, A.S. and El Said, M. 2009. Interferon gamma gene polymorphism as a biochemical marker in Egyptian atopic patients. J Investig Allergol Clin Immunol. 19: 292-298.

Hussein, Y.M., Ahmad, A.S., Ibrahem, M.M., Elsherbeny, H.M., Shalaby, S.M., El-Shal, A.S. and Sabbah, N.A. 2011. Interleukin 13 receptors as biochemical markers in atopic patients. J Investig Allergol Clin Immunol. 21: 101-107.

Irvine, A.D. 2007. Fleshing out filaggrin phenotypes. J Invest Dermatol. 127: 504-507.

Kabesch, M., Carr, D., Weiland, S.K. and von Mutius, E. 2004. Association between polymorphisms in serine protease inhibitor, kazal type 5 and asthma phenotypes in a large German population sample. Clin Exp Allergy 2004; 34: 340-345.

Kang, T.W., Lee, J.S., Oh, S.W. and Kim, S.C. 2009. Filaggrin mutation c.3321delA in a Korean patient with ichthyosis vulgaris and atopic dermatitis. Dermatol. 218: 186-187.

Kato, A., Fukai, K., Oiso, N., Hosomi, N., Murakami, T. and Ishii, M. 2003. Association of SPINK5 gene polymorphisms with atopic dermatitis in the Japanese population. Br J Dermatol. 148: 665-669.

Kent, W.J. 2002. BLAT--the BLAST-like alignment tool. Genome Res. 12: 656-664.

Kim, E., Lee, J.E., Namkung, J.H., Kim, P.S., Kim, S., Shin, E.S., Cho, E.Y. and Yang, J.M. 2009. Single nucleotide polymorphisms and the haplotype in the DEFB1 gene are associated with atopic dermatitis in a Korean population. J Dermatol Sci. 54: 25-30.

Lesiak, A., Kuna, P., Zakrzewski, M., van Geel, M., Bladergroen, R.S., Przybylowska, K., Stelmach, I., Majak, P., Hawro, T., Sysa-Jedrzejowska, A. and Narbutt, J. 2011. Combined occurrence of filaggrin mutations and IL-10 or IL-13 polymorphisms predisposes to atopic dermatitis. Exp Dermatol. 20: 491-495.

Lindblad-Toh, K., Wade, C.M., Mikkelsen, T.S., Karlsson, E.K., Jaffe, D.B., Kamal, M., Clamp, M., Chang, J.L., Kulbokas, E.J., 3rd, Zody, M.C., Mauceli, E., Xie, X., Breen, M., Wayne, R.K., Ostrander, E.A., Ponting, C.P., Galibert, F., Smith, D.R., DeJong, P.J., Kirkness, E., Alvarez, P., Biagi, T., Brockman, W., Butler, J., Chin, C.W., Cook, A., Cuff, J., Daly, M.J., DeCaprio, D., Gnerre, S., Grabherr, M., Kellis, M., Kleber, M., Bardeleben, C., Goodstadt, L., Heger, A., Hitte, C., Kim, L., Koepfli, K.P., Parker, H.G., Pollinger, J.P., Searle, S.M., Sutter, N.B., Thomas, R., Webber, C., Baldwin, J., Abebe, A., Abouelleil, A., Aftuck, L., Ait-Zahra, M., Aldredge, T., Allen, N., An, P., Anderson, S., Antoine, C., Arachchi, H., Aslam, A., Ayotte, L., Bachantsang, P., Barry, A., Bayul, T., Benamara, M., Berlin, A., Bessette, D., Blitshteyn, B., Bloom, T., Blye, J., Boguslavskiy, L., Bonnet, C., Boukhgalter, B., Brown, A., Cahill, P., Calixte, N., Camarata, J., Cheshatsang, Y., Chu, J., Citroen, M., Collymore, A., Cooke, P., Dawoe, T., Daza, R., Decktor, K., DeGray, S., Dhargay, N., Dooley, K., Dorje, P., Dorjee, K., Dorris, L., Duffey, N., Dupes, A., Egbiremolen, O., Elong, R., Falk, J., Farina, A., Faro, S., Ferguson, D., Ferreira, P., Fisher, S., FitzGerald, M., Foley, K., Foley, C., Franke, A., Friedrich, D., Gage, D., Garber, M., Gearin, G., Giannoukos, G., Goode, T., Goyette, A., Graham, J., Grandbois, E., Gyaltsen, K., Hafez, N., Hagopian, D., Hagos, B., Hall, J., Healy, C., Hegarty, R., Honan, T., Horn, A., Houde, N., Hughes, L., Hunnicutt, L., Husby, M., Jester, B., Jones, C., Kamat, A., Kanga, B., Kells, C., Khazanovich, D., Kieu, A.C., Kisner, P., Kumar, M., Lance, K., Landers, T., Lara, M., Lee, W., Leger, J.P., Lennon, N.,

Leuper, L., LeVine, S., Liu, J., Liu, X., Lokyitsang, Y., Lokyitsang, T., Lui, A., Macdonald, J., Major, J., Marabella, R., Maru, K., Matthews, C., McDonough, S., Mehta, T., Meldrim, J., Melnikov, A., Meneus, L., Mihalev, A., Mihova, T., Miller, K., Mittelman, R., Mlenga, V., Mulrain, L., Munson, G., Navidi, A., Naylor, J., Nguyen, T., Nguyen, N., Nguyen, C., Nicol, R., Norbu, N., Norbu, C., Novod, N., Nyima, T., Olandt, P., O'Neill, B., O'Neill, K., Osman, S., Oyono, L., Patti, C., Perrin, D., Phunkhang, P., Pierre, F., Priest, M., Rachupka, A., Raghuraman, S., Rameau, R., Ray, V., Raymond, C., Rege, F., Rise, C., Rogers, J., Rogov, P., Sahalie, J., Settipalli, S., Sharpe, T., Shea, T., Sheehan, M., Sherpa, N., Shi, J., Shih, D., Sloan, J., Smith, C., Sparrow, T., Stalker, J., Stange-Thomann, N., Stavropoulos, S., Stone, C., Stone, S., Sykes, S., Tchuinga, P., Tenzing, P., Tesfaye, S., Thoulutsang, D., Thoulutsang, Y., Topham, K., Topping, I., Tsamla, T., Vassiliev, H., Venkataraman, V., Vo, A., Wangchuk, T., Wangdi, T., Weiand, M., Wilkinson, J., Wilson, A., Yadav, S., Yang, S., Yang, X., Young, G., Yu, Q., Zainoun, J., Zembek, L., Zimmer, A. and Lander, E.S. 2005. Genome sequence, comparative analysis and haplotype structure of the domestic dog. Nature. 438: 803-819.

Liu, Q., Xia, Y., Zhang, W., Li, J., Wang, P., Li, H., Wei, C. and Gong, Y. 2009. A functional polymorphism in the SPINK5 gene is associated with asthma in a Chinese Han population. BMC Med Genet. 10: 59.

Marsella, R. and Girolomoni, G. 2009. Canine models of atopic dermatitis: a useful tool with untapped potential. J Invest Dermatol. 129: 2351-2357.

McGrath, J.A. 2008. Filaggrin and the great epidermal barrier grief. Australas J Dermatol. 49: 67-73; quiz 73-74.

Morar, N., Cookson, W.O., Harper, J.I. and Moffatt, M.F. 2007. Filaggrin mutations in children with severe atopic dermatitis. J Invest Dermatol. 127: 1667-1672.

Mrabet-Dahbi, S., Dalpke, A.H., Niebuhr, M., Frey, M., Draing, C., Brand, S., Heeg, K., Werfel, T. and Renz, H. 2008. The Toll-like receptor 2 R753Q mutation modifies cytokine production and Toll-like receptor expression in atopic dermatitis. J Allergy Clin Immunol. 121: 1013-1019.

Namkung, J.H., Lee, J.E., Kim, E., Kim, S., Kim, S., Shin, E.S., Cho, E.Y. and Yang, J.M. 2010[a]. Association of single nucleotide polymorphisms in the IL-12 (IL-12A and B) and IL-12 receptor (IL-12Rbeta1 and beta2) genes and gene-gene interactions with atopic dermatitis in Koreans. J Dermatol Sci. 57: 199-206.

Namkung, J.H., Lee, J.E., Kim, E., Byun, J.Y., Kim, S., Shin, E.S., Cho, E.Y. and Yang, J.M. 2010[b]. Hint for association of single nucleotide polymorphisms and haplotype in SPINK5 gene with atopic dermatitis in Koreans. Exp Dermatol. 19: 1048-1053.

Nemoto-Hasebe, I., Akiyama, M., Nomura, T., Sandilands, A., McLean, W.H. and Shimizu, H. 2009. FLG mutation p.Lys4021X in the C-terminal imperfect filaggrin repeat in Japanese patients with atopic eczema. Br J Dermatol. 161: 1387-1390.

Nishio, Y., Noguchi, E., Ito, S., Ichikawa, E., Umebayashi, Y., Otsuka, F. and Arinami, T. 2001. Mutation and association analysis of the interferon regulatory factor 2 gene (IRF2) with atopic dermatitis. J Hum Genet. 46: 664-667.

Nishio, Y., Noguchi, E., Ito, S., Ichikawa, E., Umebayashi, Y., Otsuka, F. and Arinami, T. 2003. Association between polymorphisms in the SPINK5 gene and atopic dermatitis in the Japanese. Genes Immun. 4: 515-517.

Oh, D.Y., Schumann, R.R., Hamann, L., Neumann, K., Worm, M. and Heine, G. 2009. Association of the toll-like receptor 2 A-16934T promoter polymorphism with severe atopic dermatitis. Allergy. 64: 1608-1615.

Olivry, T., DeBoer, D.J., Favrot, C., Jackson, H.A., Mueller, R.S., Nuttall, T. and Prélaud, P. 2010. Treatment of canine atopic dermatitis: 2010 clinical practice guidelines from the International Task Force on Canine Atopic Dermatitis. Vet Dermatol. 21: 233-248.

Olivry, T., Naydan, D.K. and Moore, P.F. 1997. Characterization of the cutaneous inflammatory infiltrate in canine atopic dermatitis. Am J Dermatopathol. 19: 477-486.

Palmer, C.N., Irvine, A.D., Terron-Kwiatkowski, A., Zhao, Y., Liao, H., Lee, S.P., Goudie, D.R., Sandilands, A., Campbell, L.E., Smith, F.J., O'Regan, G.M., Watson, R.M., Cecil, J.E., Bale, S.J., Compton, J.G., DiGiovanna, J.J., Fleckman, P., Lewis-Jones, S., Arseculeratne, G., Sergeant, A., Munro, C.S., El Houate, B., McElreavey, K., Halkjaer, L.B., Bisgaard, H., Mukhopadhyay, S. and McLean, W.H. 2006. Common loss-of-function variants of the epidermal barrier protein filaggrin are a major predisposing factor for atopic dermatitis. Nat Genet. 38: 441-446.

Rawlings, A.V. and Matts, P.J. 2005. Stratum corneum moisturization at the molecular level: an update in relation to the dry skin cycle. J Invest Dermatol. 124: 1099-1110.

Rawlings, A.V., Scott, I.R., Harding, C.R. and Bowser, P.A. 1994. Stratum corneum moisturization at the molecular level. J Invest Dermatol. 103: 731-741.

Rhodes, K.H., Kerdel, F. and Soter, N.A. 1987. Comparative aspects of canine and human atopic dermatitis. Semin Vet Med Surg (Small Anim). 2: 166-172.

Roduit, C., Wohlgensinger, J., Frei, R., Bitter, S., Bieli, C., Loeliger, S., Büchele, G., Riedler, J., Dalphin, J.C., Remes, S., Roponen, M., Pekkanen, J., Kabesch, M., Schaub, B., von Mutius, E., Braun-Fahrländer, C. and Lauener, R. 2011. Prenatal animal contact and gene expression of innate immunity receptors at birth are associated with atopic dermatitis. J Allergy Clin Immunol. 127: 179-185, 185.e1.

Rozen, S. and Skaletsky, H.J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and

Protocols: Methods in Molecular Biology. S. Krawetz and S. Misener (eds.). New Jersey: Humana Press: 365-386.

Sandilands, A., Terron-Kwiatkowski, A. and Hull, P.R. 2007. Comprehensive analysis of the gene encoding filaggrin uncovers prevalent and rare mutations in ichthyosis vulgaris and atopic eczema. Nat Genet. 39: 650-654.

Simon, M., Haftek, M., Sebbag, M., Montézin, M., Girbal-Neuhauser, E., Schmitt, D. and Serre, G. 1996. Evidence that filaggrin is a component of cornified cell envelopes in human plantar epidermis. Biochem J. 317:173-177.

Smith, F.J., Irvine, A.D. and Terron-Kwiatkowski, A. 2006. Loss-of-function mutations in the gene encoding filaggrin cause ichthyosis vulgaris. Nat Genet. 38: 337-342.

Vasilopoulos, Y., Sharaf, N., di Giovine, F., Simon, M., Cork, M.J., Duff, G.W. and Tazi-Ahnini, R. 2011. The 3'-UTR AACCins5874 in the stratum corneum chymotryptic enzyme gene (SCCE/KLK7), associated with atopic dermatitis; causes an increased mRNA expression without altering its stability. J Dermatol Sci. 61: 131-133.

Vonholdt, B.M., Pollinger, J.P., Lohmueller, K.E., Han, E., Parker, H.G., Quignon, P., Degenhardt, J.D., Boyko, A.R., Earl, D.A., Auton, A., Reynolds, A., Bryc, K., Brisbin, A., Knowles, J.C., Mosher, D.S., Spady, T.C., Elkahloun, A., Geffen, E., Pilot, M., Jedrzejewski, W., Greco, C., Randi, E., Bannasch, D., Wilton, A., Shearman, J., Musiani, M., Cargill, M., Jones, P.G., Qian, Z., Huang, W., Ding, Z.L., Zhang, Y.P., Bustamante, C.D., Ostrander, E.A., Novembre, J., Wayne, R.K. 2010. Genome-wide SNP and haplotype analyses reveal a rich history underlying dog domestication. Nature. 464: 898-902.

Weidinger, S., Illig, T., Baurecht, H., Irvine, A.D., Rodriguez, E., Diaz-Lacava, A., Klopp, N., Wagenpfeil, S., Zhao, Y., Liao, H., Lee, S.P., Palmer, C.N., Jenneck, C., Maintz, L., Hagemann, T., Behrendt, H., Ring, J., Nothen, M.M., McLean, W.H. and Novak, N. 2006. Loss-of-function variations within the filaggrin gene predispose for atopic dermatitis with allergic sensitizations. J Allergy Clin Immunol. 118: 214-219.

Willemse, T. 1988. Comparative aspects of canine and human atopic dermatitis. Semin Vet Med Surg (Small Anim). 3: 255.

Wood, S.H., Ollier, W.E., Nuttall, T., McEwan, N.A. and Carter, S.D. 2010. Despite identifying some shared gene associations with human atopic dermatitis the use of multiple dog breeds from various locations limits detection of gene associations in canine atopic dermatitis. Vet Immunol Immunopathol. 138: 193-197.

Zhang, H., Guo, Y., Wang, W., Shi, M., Chen, X and Yao, Z. 2011. Mutations in the filaggrin gene in Han Chinese patients with atopic dermatitis. Allergy. 66: 420-427.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. 2000. A greedy algorithm for aligning DNA sequences. J Comput Biol. 7: 203-214.