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

Gunnaporn Suriyaphol*  Prapat Suriyaphol  Meena Sarikaputi  Sirin Theerawatanasirikul  Achariya Sailasuta

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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Original Article

บทความ

ความสัมพันธ์ของความหลากหลายทางพันธุกรรมของยีนฟิแลกกรินกับโรคผิวหนังชนิดอะโทปีในสุนัข

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

ฟิแลกกรินเป็นโปรตีนในกลุ่มคอร์นิไฟด์เอนเวลโลปซึ่งมีความสําคัญในการสร้างและค้ําจุนโครงสร้างผิวหนังชั้นนอก ดังกล่าวกับการเกิดโรคผิวหนังชนิดอะโทปีในสุนัขพันธุ์ลาบราดอร์รีทริฟเวอร์ในสหราชอาณาจักร มีรายงานการพบความสัมพันธ์ของสนิป (single nucleotide polymorphism, SNP) หรือความหลากหลายทางพันธุกรรมจากความแตกต่างของลำดับนิวคลีโอไทด์เบสเพียง 1 ตําแหน่งของยีนฟิแลกกรินที่ตําแหน่ง 64,297,022 (อาร์เอส 22588227) บนโครโมโซมที่ 17 ของสุนัขกับโรคผิวหนังชนิดอะโทปีของสุนัขพันธุ์ลาบราดอร์รีทริฟเวอร์ในสหราชอาณาจักร ในการศึกษาครั้งนี้ความแตกต่างของตําแหน่งนี้เป็นตัวแปรที่ผ่านการทดสอบในกลุ่มตัวอย่างที่ประกอบด้วยสุนัขป่วยและสุนัขสุขภาพดี โดยไม่พบความแตกต่างของสนิปที่ตําแหน่งดังกล่าวในสุนัขที่ป่วยและสุนัขปกติ แต่พบสนิปใหม่ 13 ตําแหน่งในยีนฟิแลกกรินและพบข้อข้อยู่ที่ตําแหน่งนี้ไม่ปรากฏในฐานข้อมูลจีโนมของสุนัขจากการศึกษาที่พบความถี่อัลลีลของสนิปที่ตําแหน่ง 64,297,000 (p = 0.041, อัตราส่วนออด = 3.920) และ 64,297,126 (p = 0.043, อัตราส่วนออด = 3.706) อาจมีความสัมพันธ์กับความสามารถในการเกิดโรคผิวหนังชนิดอะโทปี ซึ่งต่างจากตําแหน่งของสนิปที่ตําแหน่งนี้ในกลุ่มทดลองและกลุ่มควบคุม ที่ก็จะพบไม่ได้ แสดงว่าการพบอยู่ของสนิปนี้ที่ตําแหน่งนี้มีความสัมพันธ์กับความสามารถในการเกิดโรคผิวหนังชนิดอะโทปี ซึ่งแสดงว่าสนิปนี้มีความสัมพันธ์กับความสามารถในการเกิดโรคผิวหนังชนิดอะโทปีในสุนัขพันธุ์ลาบราดอร์รีทริฟเวอร์ ดังกล่าวกับการเกิดโรคผิวหนังชนิดอะโทปีในสุนัขพันธุ์เล็กจำนวน 41 ตัว ประกอบด้วยพันธุ์พุดเดิ้ล 21 ตัว พันธุ์ชิสุห์ 3 ตัว และพันธุ์ปั๊ก 3 ตัว โดยมีตัวอย่าง 12 ตัวซึ่งเป็นตัวอย่างที่ไม่ผ่านการเกิดโรคเป็นตัวอย่างที่ไม่ผ่านการเกิดโรค รายละเอียดเพิ่มเติมเกี่ยวกับการศึกษาครั้งนี้จะนำเสนอในบทความต่อไป

คำสําคัญ: โรคผิวหนังชนิดอะโทปีในสุนัข สุนัข ฟิแลกกริน สนิป

Introduction

Canine atopic dermatitis (CAD) is defined as “a genetically-predispositional 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 (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; Kubesch et al. 2004; Liu et al., 2009; Namkung et al., 2010b), kallikrein-related peptidase 7 or stratum corneum chymotryptic enzyme (KLK 7/SCCE)
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; Sandlildans 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°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°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 Skaltsky, 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 MgSO4, 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°C for 2 min; 40 cycles of 94°C for 15 sec, 53°C for 30 sec and 68°C for 3 min; and one cycle of 68°C for 5 min. After the amplification reaction, the samples were held at -20°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>
<thead>
<tr>
<th>Sequences (5’ → 3’)</th>
<th>Locations</th>
<th>Lengths of PCR products (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fwd outer P’</td>
<td>CTTTGTTCAGGATATTTCACCA</td>
<td>64,294,986</td>
</tr>
<tr>
<td>Rev outer P’</td>
<td>TTAACAGCACCCTTGTGAGATTC</td>
<td>64,297,374</td>
</tr>
<tr>
<td>Fwd inner-1 P’</td>
<td>TGTGTGGGTTCATATTCCTACAA</td>
<td>64,295,788</td>
</tr>
<tr>
<td>Rev inner P’</td>
<td>GTCCAGTCCAGTCAGAGGA</td>
<td>64,296,667</td>
</tr>
<tr>
<td>Fwd inner-2 P’</td>
<td>TGTCGTCTACGGGTGCTGAGA</td>
<td>64,296,827</td>
</tr>
</tbody>
</table>

**Table 1 Primer sequences and their locations**
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 haplotype block 3.3 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 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 22% frequencies (Table 3). Two haplotype block patterns (GACCGGTTTTTCGGC 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 (GACCGGTTTTTCGGC) 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

<table>
<thead>
<tr>
<th>Chromosomal position (bp)</th>
<th>Alleles</th>
<th>Alternative allele frequency, n(%)</th>
<th>P value</th>
<th>OR (CGD) (95%CI)</th>
<th>RR (CGD) (95%CI)</th>
<th>OR (Alt) (95%CI)</th>
<th>RR (Alt) (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>64,296,925</td>
<td>A G</td>
<td>21(20.588) 58(56.000)</td>
<td>0.265</td>
<td>0.400 (0.116-1.405)</td>
<td>0.478 (0.157-1.288)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,000</td>
<td>C A</td>
<td>21(20.588) 50(49.020)</td>
<td>0.041**</td>
<td>0.270 (0.075-0.881)</td>
<td>0.343 (0.107-0.907)</td>
<td>3.706 (1.136-13.335)</td>
<td>0.478 (0.157-1.288)</td>
</tr>
<tr>
<td>64,297,126</td>
<td>A C</td>
<td>21(20.588) 48(47.529)</td>
<td>0.043**</td>
<td>0.270 (0.079-0.933)</td>
<td>0.343 (0.112-0.948)</td>
<td>3.706 (1.072-12.622)</td>
<td>0.478 (0.157-1.288)</td>
</tr>
<tr>
<td>64,297,148</td>
<td>C G</td>
<td>3(2.941) 24(23.529)</td>
<td>0.112</td>
<td>3.111 (0.895-10.645)</td>
<td>0.919-7.697 (0.094-1.117)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,153</td>
<td>G A</td>
<td>3(2.941) 26(25.490)</td>
<td>0.069</td>
<td>0.919-7.697 (0.094-1.117)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
<td>0.478 (0.157-1.288)</td>
</tr>
<tr>
<td>64,297,154</td>
<td>C T</td>
<td>3(2.941) 25(24.510)</td>
<td>0.071</td>
<td>0.919-7.697 (0.094-1.117)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
<td>0.478 (0.157-1.288)</td>
</tr>
<tr>
<td>64,297,171</td>
<td>G T</td>
<td>19(18.627) 47(46.078)</td>
<td>0.142</td>
<td>0.952-11.279 (0.300-3.102)</td>
<td>0.963-8.094 (0.300-3.102)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,173</td>
<td>C T</td>
<td>19(18.627) 49(48.039)</td>
<td>0.215</td>
<td>0.156-1.281 (0.050-0.454)</td>
<td>0.213-1.207 (0.050-0.454)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,222</td>
<td>G T</td>
<td>19(18.627) 49(48.039)</td>
<td>0.215</td>
<td>0.156-1.281 (0.050-0.454)</td>
<td>0.213-1.207 (0.050-0.454)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,142*</td>
<td>T C</td>
<td>3(2.941) 20(19.608)</td>
<td>0.265</td>
<td>0.688-8.335 (0.203-2.308)</td>
<td>0.756-6.207 (0.203-2.308)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,148*</td>
<td>C G</td>
<td>3(2.941) 22(21.569)</td>
<td>0.175</td>
<td>0.788-9.448 (0.230-2.727)</td>
<td>0.836-9.633 (0.230-2.727)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
<tr>
<td>64,297,153*</td>
<td>G A</td>
<td>3(2.941) 22(21.569)</td>
<td>0.175</td>
<td>0.788-9.448 (0.230-2.727)</td>
<td>0.836-9.633 (0.230-2.727)</td>
<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
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<tr>
<td>64,297,154*</td>
<td>C T</td>
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<td>2.500 (0.712-8.641)</td>
<td>2.091 (0.777-6.366)</td>
</tr>
</tbody>
</table>

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 tzu 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 r² 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.
the identification of the unique fragment, this study is composed of several repeats which obstruct since FLG nucleotide sequence in the canine genome should further be investigated. Since Poodles with CAD and the block homozygosity (Table 4). The close relationship between Poodles with atopy had the same pattern, ACAGATGCGCGAT, 22.5%), we found that all 8 (GACCGCTTTTCGC, 60.8% and the two highest percent frequencies addition, from the analysis of 2 haplotype blocks with polymorphism was observed in FLG gene. In support the role of FLG in CAD since the significant p-values 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

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References


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

<table>
<thead>
<tr>
<th>Haplotype block</th>
<th>Poodles</th>
<th>Control</th>
<th>Shih tzu</th>
<th>CAD</th>
<th>Control</th>
<th>CAD</th>
<th>Pugs</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. GACCGCTTTTCGC</td>
<td>100.0</td>
<td>82.6</td>
<td>0.0</td>
<td>60.0</td>
<td>40.0</td>
<td>0.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>2. ACAGATGCGCGAT</td>
<td>0.0</td>
<td>39.0</td>
<td>100.0</td>
<td>0.0</td>
<td>50.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>

*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

<table>
<thead>
<tr>
<th>Haplotypes</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. GACCGCTTTTCGC</td>
<td>60.8</td>
</tr>
<tr>
<td>2. ACAGATGCGCGAT</td>
<td>22.5</td>
</tr>
<tr>
<td>3. GACCGCTTTTCGC</td>
<td>2.0</td>
</tr>
<tr>
<td>4. GAGCGCGCGTCGC</td>
<td>2.9</td>
</tr>
<tr>
<td>5. GACCGCTTTTCGC</td>
<td>2.0</td>
</tr>
<tr>
<td>6. GGGCGCGCTTCGC</td>
<td>2.0</td>
</tr>
<tr>
<td>7. GCAGATGCGGTCGC</td>
<td>2.0</td>
</tr>
</tbody>
</table>

In order to search for new SNP mutations, we aligned DNA fragments of 2,288 bp to draft dog genome databases, using both BLAST (NCBI 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 are plausibly associated with CAD. Our data found association between two new SNPs in the FLG gene and the CAD, but we included 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.

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