



Haplotype sharing excludes canine orthologous *Filaggrin* locus in atopy in West Highland White Terriers

J. Barros Roque*, C. A. O'Leary*, M. Kyaw-Tanner*, M. Latter*, K. Mason[†], M. Shipstone[‡], L. Vogelneust[§] and D. L. Duffy[¶]

*School of Veterinary Science, The University of Queensland, St Lucia Queensland 4072, Australia. [†]Dermcare, 22 Aranda St, Springwood, Queensland 4127, Australia. [‡]Veterinary Specialist Services, 263 Appleby Rd, Stafford Heights, Queensland 4053, Australia. [§]The University of Sydney, University Veterinary Teaching Hospital, Camden New South Wales 2570, Australia.

[¶]Genetic Epidemiology Laboratory, Queensland Institute of Medical Research, Herston Queensland 4029, Australia

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Source/description: Atopic dermatitis (atopy) is an inherited, allergic skin disorder of dogs¹ and humans,² and is common in West Highland White Terriers (WHWT).³ Null mutations in *Filaggrin* (*FLG*) occur in 18–48% of humans with atopic dermatitis, compared with 7% of the general population.⁴ *FLG* encodes a protein involved in epidermal barrier maintenance,⁴ with impaired epidermal function contributing to the development of canine atopic dermatitis⁵ by enhancing antigen presentation to the immune system.⁶ The role of *flg* in atopy in WHWT was examined.

Methods: The gDNA was extracted from EDTA blood from 49 WHWT with atopy and 30 without atopy (Table S1) with a QIAamp Blood Midi Kit (Qiagen GmbH, Hilden, Germany), saline extraction,⁷ or phenol extraction.⁸ Canine *flg* was localized using BLASTN (<http://www.ensembl.org>) to CFA17 at 64.296–64.305 Mbp (genome build CANFAM2.0 May 2006). Fifteen genetic markers nearby were identified (Tables S2 & S3). Primers were supplied by GeneWorks (Adelaide, Australia). M13-labelling was used (Tables S4–S6),⁹ and genotyping was performed on a 3130xl Genetic Analyzer (Hitachi/Applied Biosystems, Foster City, CA, USA), using POP 7 Polymer, size standard GENESCAN 500 Liz or GENESCAN 1200 Liz (ABI Biosystems, Foster City, CA, USA). Analysis was carried out using GENEMAPPER.

Preliminary pedigree analyses were carried out using Sib-pair,¹⁰ including testing for Mendelian errors. A segregation analysis was carried out using MENDEL 9.0,¹¹ RELPAIR¹² confirmed the recorded pedigree information for first degree relationships from the marker data. Haplotypes were constructed using SIMWALK2,¹³ and used for association and non-parametric linkage analysis in Sib-pair. Association analysis was carried out using a simulation-based method. Multipoint non-parametric and parametric linkage analyses were performed using SIMWALK2 and SUPERLINK,¹⁴ in the latter maximizing over fully penetrant dominant and recessive models, assuming 50% and 90% allele frequencies in founders. The markers were ordered using positions in the canine sequence and confirmed using Mendel.

Results: As a result of complex ascertainment, the dataset was not very informative for segregation analysis of atopy. Observed inheritance patterns were equally consistent with a common fully penetrant dominant (risk allele frequency $P_D = 0.5$, model

log likelihood $LL = -19.28$) or recessive ($P_D = 0.9$, $LL = -18.91$) major locus.

The observed intermarker recombination was consistent with the chosen genetic marker map (Table S2). There were no observed recombinants among the fine-mapping markers genotyped close to *flg* (two-point lod scores: M1–M4, maximum lod score $Z_{\max} = 5.72$ at recombination fraction $c = 0$; M4–M8, $Z_{\max} = 4.02$ at $c = 0$). Haplotypes were constructed (Table S7). The haplotype frequencies did not differ significantly between affected and unaffected animals ($P = 0.29$). Non-parametric linkage analysis failed to demonstrate linkage of the microsatellite marker haplotypes to atopy (affecteds-only analysis $P = 0.95$; general pairs method $P = 0.45$). On parametric linkage analysis under the dominant and recessive models, the LOD score for atopy to the haplotype (at $c = 0$) was $LOD = -0.4$.

Comments: This study excludes a large causative role for the canine *flg* orthologue in atopy in WHWT. We had expected that atopic disease in WHWT was likely to be monogenic, or to perhaps oligogenic, due either to a founder mutation or hitchhiking with a breeder-selected trait. Under either of these hypotheses and high trait prevalence, we would expect the gene to be highly penetrant and relatively common, thus displaying co-segregation of flanking marker haplotypes with disease. If such a haplotype was measured, our association analysis would have 100% power to detect its effect if fully penetrant, and 55% power if the penetrance was 50%. We therefore cannot exclude a role for mutations with small effect sizes.

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Correspondence: C. O'Leary (c.oleary@uq.edu.au)

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Diagnostic criteria for selection of dogs in this study.

Table S2 Information on 8 published genetic markers used in the study.

Table S3 Microsatellite details and amplification primer sequences for three newly identified microsatellites (M1, M4, M5) using Tandom Repeats Finder http://research.nhgri.nih.gov/dog_genome/ and four from <http://www.vgl.ucdavis.edu/dogset/> (M8, M9, M11, M12) used in this study.

Table S4 Amplification conditions and PCR components in a 10- μ l reaction volume using Multiplex PCR Kit (Qiagen GmbH, Germany).

Table S5 Thermocycling protocol for microsatellites *FH3369*, *PEZ8*, *FH3995*, *REN240A05* (multiplex 3) and *FH3047*, *REN294E18*, *FH4023* and *FH2869* (multiplex 4).

Table S6 Thermocycling Protocol for *M1*, *M4* and *M5* (multiplex 1), *M8*, *M9*, *M11* and *M12* (multiplex 2).

Table S7 Haplotypes constructed using microsatellite markers (*M12-M11-M9-M1-M4-M8*) in the region of the canine *flg* orthologue.

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Table S1 . Diagnostic criteria for selection of dogs in the present study.

Group of dogs	Diagnostic Criteria
Atopic dogs (n = 34)	Diagnosed as atopic by veterinary dermatologist ¹⁵
Likely atopic (n =15)	No owner history of atopy but; - clinical signs (eg pruritus especially affecting the feet, face or ears, erythema, alopecia, lichenification, hyperpigmentation or salivary staining of the coat) compatible with atopy - or history of more than one episode of otitis externa, pyoderma or yeast infection - or history of moderate to severe pruritus on any part of the body
Non-atopic (n = 19)	From a line of West Highland White Terriers minimally affected by atopy, and at least 5 years old No history or clinical symptoms of atopy, pyoderma, aural disease, pruritis, foot chewing or face rubbing.
Likely non-atopic (n = 11)	Diagnosis as for non-atopic and while no skin changes consistent with atopy; - had either one episode of otitis externa and/or pyoderma (one dog had two episodes of antibiotic responsive non-pruritic pyoderma) - or occasional mild pruritis on any part of the body.

Table S2. Information on 8 published genetic markers used in the study (http://research.nhgri.nih.gov/dog_genome/).

Marker	Position (Mbp)	No. dogs	Observed alleles (Frequency) ^π	HWE-P ^ρ
<i>REN240A05</i>	6.781200	29	238 (0.091) 240 (0.909) 320 (0.0001)	0.69
<i>FH3369</i>	11.952950	27	313 (0.0001) 322 (0.046) 324 (0.046) 326 (0.091) 330 (0.636) 334 (0.182)	0.38
<i>FH3047</i>	18.072730	28	219 (0.932) 222 (0.068)	0.57
<i>REN294E18</i>	26.840809	28	314 (0.591) 318 (0.046) 320 (0.273) 324 (0.091)	0.87
<i>FH4023</i>	40.281337	28	464 (0.091) 473 (0.909) 477 (0.0001)	0.69
<i>FH3995</i>	45.917039	78	355 (0.034) 358 (0.067) 362 (0.0001) 366 (0.295) 370 (0.034) 414 (0.0001) 418 (0.034) 437 (0.0001) 438 (0.0001) 448 (0.436) 449 (0.0001) 453 (0.067) 456 (0.0001) 457 (0.0001) 460 (0.033) 464 (0.0001)	0.11
<i>PEZ8</i>	62.562738	29	243 (0.046) 251 (0.136) 255 (0.818)	0.35
<i>M12</i>	64.077887	76	293 (0.0001) 295 (0.467) 299 (0.534)	0.98
<i>M11</i>	64.154009	76	371 (0.928) 375 (0.0001) 387 (0.072) 392 (0.0001)	0.94

<i>M9</i>	64.185383	76	296 (0.974) 300 (0.026)	0.28
<i>M5</i>	64.245151	78	653 (0.013) 656 (0.096) 660 (0.199) 664 (0.013) 669 (0.244) 673 (0.353) 677 (0.026) 681 (0.032)	0.31
<i>M1</i>	64.315320	79	169 (0.892) 173 (0.108)	0.73
<i>M4</i>	64.315892	79	256 (0.892) 258 (0.108)	0.73
<i>M8</i>	64.490392	76	332 (0.0001) 334 (0.072) 336 (0.928)	0.71
<i>FH2869</i>	67.210932	28	220 (0.781) 222 (0.219)	0.76

^{TT} maximum likelihood estimates for founders
^p Hardy-Weinberg Equilibrium

Table S3. Microsatellite details and amplification primer sequences for three newly identified microsatellites (M1, M4, M5) using Tandem Repeats Finder http://research.nhgri.nih.gov/dog_genome/ and four from <http://www.vgl.ucdavis.edu/dogset/> (M8, M9, M11, M12) used in the present study. Unavailable primers sequences were designed using Primer3 (http://www.genome.wi.mit.edu/genome_software/other/primer3.html).

Microsatellite	Type	Location in CFA 17 (bp)	Forward Primer	Reverse Primer	Product Size (bp)
M12	Di (CT) _n	64,077,887	AGCTTGGGGTTGGAAATCA	TCATTAGAAATTTGCAGCTCACC	293-299
M11	Tetra (TAAA) _n	64,154,009	GCTCTGAGGCATCATCCTTT	TCTCCATCTAGCCTTGGAAACA	371-392
M9	Tetra (TAAA) _n	64,185,383	AAC TTGAGGGGACTGGTC A	TCCCACAGGTAATTGCTCTCA	296-300
M5	Tetra (TTTC) _n	64,245,151	AAACCACCCATCCCTCTACC	ACAGCCTCTGAGTTGCAGGT	659-681
M1	Di (TG) _n	64,315,320	TTGCCCTTCATTGACCTCAGA	CATTCCCTCCCTCTCTCCAT	169-173
M4	Di (CA) _n	64,315,892	ATAGTCATGCCCGAGGCACTC	CATTCCAAAGATTTCCTCCACT	256-260
M8	Di (CA) _n	64,490,392	CCTAGGCTGCTTCCTGTGCT	CCTGTGTGGGCCAAACCTAT	332-336

Table S4. Amplification conditions and PCR components in a 10µl reaction volume using Multiplex PCR Kit (QIAGEN GmbH, Germany).

Component	Concentration
Template DNA	10ng/µl
F primer	0.04 µM (M1, M4, M5) or 0.05 µM for the remaining microsatellites
R primer	0.16 µM (M1 and M5), 0.14 µM (M4) or 0.1 µM for the remaining microsatellites
M13-labelled F primer	0.2-0.3 µM

Table S5. Thermocycling protocol for microsatellites *FH3369*, *PEZ8*, *FH3995*, *REN240A05* (multiplex 3) and *FH3047*, *REN294E18*, *FH4023*, and *FH2869* (multiplex 4).

Time	Optimized temperature	Number of cycles
15 minutes	95°C	1 cycle
30 seconds	94°C	35 cycles
90 seconds	55°C	35 cycles
90 seconds	72°C	35 cycles
10 minutes	72°C	1 cycle
Overnight or long term storage	12°C or -20°C	

Table S6. Thermocycling Protocol for *M1*, *M4* and *M5* (multiplex 1), *M8*, *M9*, *M11* and *M12* (multiplex 2).

Time	Optimized temperature	Number of cycles
15 minutes	95°C	1 cycle
30 seconds	94°C	35 cycles
90 seconds	57°C	35 cycles
60 seconds	72°C	35 cycles
10 minutes	72°C	1 cycle
Overnight or long term storage	12°C or -20°C	

Table S7. Haplotypes constructed using microsatellite markers

(*M12-M11-M9-M1-M4-M8*) in the region of the canine *flg* orthologue. There were no detectable intermarker recombinants within this 412-kbp interval. The group differences were not significant using a gene-dropping simulation-based test.

Haplotype		Number of chromosomes	
		Atopic dogs	Nonatopic dogs
A	295371296169256336	31 (.470)	35 (.921)
B	299371296169256336	18 (.273)	2 (.053)
C	293392296173258334	1 (.016)	1 (.025)
D	299371300169256336	7 (.109)	0 (.000)
E	299387296173258336	2 (.031)	0 (.000)
F	299371296169256332	0 (.000)	0 (.000)
G	299375296169256336	1 (.016)	0 (.000)
H	293392296173258336	1 (.016)	0 (.000)
I	295371296169256334	3 (.047)	0 (.000)
J	295387296173256336	1 (.016)	0 (.000)
K	299371296169258336	1 (.016)	0 (.000)