Variation in ovine RAS Guanyl Releasing Protein 1 (RASGRP1) gene and its association with flystrike in New Zealand sheep

L. E. R. Burrows[1], H. Zhou[1], C. M. A. Frampton[3], R. H. J. Forrest[2], J. G. H. Hickford[1]

[1] Gene-Marker Laboratory, Faculty of Agriculture and Life Sciences, PO Box 85084, Lincoln University, Lincoln 7647, Christchurch, New Zealand
[2] Faculty of Education Humanities and Health Science, Eastern Institute of Technology, Private Bag 1201, Napier 4142, New Zealand
[3]Department of Medicine, University of Otago, Christchurch 8140, New Zealand

Summary

Flystrike is a parasitic disease that results in major cost to the sheep industry both nationally and worldwide. In recent years, research has focused on breeding sheep that are less susceptible to flystrike. Previous research has identified the Ras Guanyl Releasing Protein 1 gene (*RASGRP1*) as a possible candidate gene for flystrike resistance. To investigate this candidate gene, 791 blood samples were collected over five years from sheep with and without flystrike (n =421 with and n = 370 without), and of a variety of breeds. DNA from these blood samples was subjected to Polymerase Chain Reaction – Single Strand Conformational Polymorphism (PCR-SSCP) analysis using primers that targeted exon 16 of *RASGRP1*. Three variant sequences (*A*-*C*) were found and the presence of *A* was revealed to influence flystrike susceptibility (P = 0.015), while the presence of *C* was associated with a decrease in flystrike (P = 0.047).

Keywords: flystrike, RASGRP1, sheep

Introduction

Flystrike is a common parasitic disease of sheep with a reported prevalence in New Zealand (NZ) ranging from 3.4% to 24% (Scobie and O'Connell, 2010), and an average prevalence of 2.15% from 2009 to 2011 (Pickering, 2013). It is estimated to cost the NZ sheep industry $60.2 million annually (Ludemann et al., 2010). Research has begun to focus on genetics to reduce flystrike in sheep, and in Australian Merino sheep flystrike resistance has been estimated to have a heritability of 0.26. Susceptibility to flystrike has a > 0.9 genetic correlation with the occurrence of fleecerot (Raadsma, 1991). Pickering (2013) identified the RAS Guanyl Releasing Protein 1 (RASGRP1) gene (*RASGRP1*), located within 100kbp of a SNP (OAR_35146905), as a potential candidate gene associated with variation in flystrike resistance. This was the first indication that *RASGRP1* might play a role in flystrike resistance in sheep.

The Ras family of proteins are involved in many cell-signally pathways, where they regulate a variety of cell functions (Dower, 2000; Fuller et al., 2012). RASGRP1 functions as a nucleotide exchange factor and activates Ras through the exchange of bound GDP for GTP (Roose, *et al*., 2005). This in turn activates several signalling pathways, including the activation of mitogen-activated protein kinases (MAPKs) (Stone, 2011), extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK), and p38 (Priatel *et al*., 2002). RASGRP1 is expressed at elevated levels in T cells, but it is also expressed in B cells, natural

killer cells and mast cells (Ebinu et al., 2000; Lee *et al.* 2009; Liu et al., 2007). It is specifically involved in the T cell receptor (TCR) signalling pathway, where it enables the generation of a diverse collection of TCRs, and these allow a range of responses to a variety of foreign peptides presented by the major histocompatibility complex (MHC) (Priatel et al., 2002).

With flystrike, Bowles et al. (1994) revealed an interaction between larval antigens and MHC class II molecules on antigen-presenting cells. Hence a gene such as *RASGRP1*, that produces a protein that is involved in immune response, may have a role in regulating flystrike susceptibility in sheep. Exons 16 and 17 of *RASGRP1,* in part make up the C-terminal tail domain, which has an essential role in RASGRP1 function (Yasuda et al., 2007; Fuller et al., 2012). In this study, variation with exon 16 of ovine *RASGRP1* was investigated along with its association, if any, with the occurrence of flystrike. Exon 16 is the largest exon in *RASGRP1* (http://www.ensembl.org).

Materials and methods

Sheep

Blood samples from 791 sheep were investigated for variation in *RASGRP1*. Of these samples, 421 were from sheep with flystrike and 370 were from sheep without flystrike and collected on the same farms as the sheep with flystrike. The samples were collected over five strike seasons (2013 to 2017), and the sheep were sourced from both commercial and stud sheep properties throughout NZ. The sheep breeds investigated included Merino, Corriedale, Perendale, Romney, Lincoln, Coopworth, Poll Dorset, Texel, Dorset Down, Suffolk, South Suffolk, Shropshire and various crossbreeds and composites.

Blood collection and DNA purification

The blood samples were collected onto FTA cards (Whatman, Middlesex, UK) by nicking the lower part of the ear with electrical side-cutters. This sampling method did not require animal ethics approval as ear nicking is considered a common farming practice. The samples were labelled with the year, farm, breed, gender and age of the sheep (in years) and whether they had flystrike or not. The cards were left to dry and were stored in darkness at room temperature until analysed. DNA from blood was purified using a two-step washing procedure described by Zhou et al. (2006).

Primer design for amplifying a region of ovine *RASGRP1*

Two PCR primers were designed to amplify a 446 bp fragment within exon 16 of ovine *RASGRP1* based on the predicted sequence of ovine *RASGRP1* (XM 012181173.2). The primers were *RASGRP1*-up (5'-ACAGCACCTGAGGAAGGA-3') and *RASGRP1*-dn (5'-ATGTTCTTCCCTTGTTAGCC-3'). These primers were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

PCR-SSCP analysis and genotyping

PCR amplification was performed in a 15 μL reaction containing the genomic DNA on one 1.2 mm punch of FTA card, 0.25 μM of each primer, 150 μM dNTP's (Bioline,

London, UK), 2.5 mM of Mg^{2+}, 0.5 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 1× the reaction buffer supplied with the enzyme. The thermal profile consisted of 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C, with a final extension of 5 min at 72 °C. Amplification was carried out in S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA).

A 0.7 µL aliquot of each amplicon was mixed with 7 µL of loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol). After denaturation at 95 °C for 5 min, the samples were rapidly cooled on wet ice and then loaded on 16 cm × 18 cm, 14% acrylamide:bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was performed using Protean II xi cells (Bio-Rad), at 300 V for 19 h at 18 °C in 0.5 × TBE buffer. Gels were silver-stained according to the method of Byun *et al*. (2009).

Sequencing of the ovine *RASGRP1* variants and sequence analysis

Amplicons from sheep that appeared to be homozygous based on the PCR-SSCP banding patterns observed, were sequenced directly by the Lincoln University DNA Sequencing Facility. DNAMAN (version 5.2.10, Lynnon BioSoft, Canada) was used for sequence alignment, translations and comparisons. The Blast algorithm was used to search the NCBI GenBank database (http://blast.nci.nlm.nih.gov/) for homologous sequences.

Statistical analysis

Variant and genotype frequencies were calculated for all sheep investigated (n = 791), and for sheep with and without flystrike (n = 421 and n = 370, respectively).

All statistical analyses were performed using SPSS_ version 24 (Chicago, IL, USA). For each animal the presence or absence of flystrike was coded with a 1 or 0, respectively. Likewise, for each *RASGRP1* variant, the presence or absence of that variant in each sheep's genotype was coded with a 1 or 0, respectively. Both univariate and multivariate analyses were undertaken. Variables considered in the latter included: age, gender, breed, geographical region, and year. Age and gender were combined into a single variable with the following groups: lambs (as gender was not recorded for most of the lambs, which were from commercial flocks), Ewe2 and Ram2 which were hoggets (sheep of one year of age) for both genders, and Ewe3 and Ram3 which were two tooths and older for both genders. Breeds were categorised into four groups: 1) Merino X which included Merinos, Corriedale's and Merino crosses (half-breds); 2) Pure-bred which included Romney, Lincoln and Perendale; 3) Cross-bred which included Composites, Coopworth, Corriedale-crosses, Perendale-crosses, Romney-crosses and commercial cross-bred sheep; 4) Black face sheep, which included all black face breeds: Dorset Downs, Suffolk, South Suffolk, Southdown, Shropshire, black-faced cross-breds and two white faced down wool breeds, the Poll Dorset and Texel. Geographical region was grouped into the three main sampling areas: North Canterbury, which included Northland as this was the only north island farm in this study, Mid Canterbury, which included Banks Peninsula, and Otago which was combined with South Canterbury.

For each gene variant, a Pearson chi-square test along with a binary logistic regression was performed to explore whether the presence or absence of the variant was associated with the presence or absence of flystrike and to determine the odds-ratio, respectively. A multivariate binary logistic regression was then performed to determine the independent effects of gene variant on incidence of flystrike when year, breed, geographical location, age

and gender were considered as factors.

Results

Three unique PCR-SSCP banding patterns (Figure 1) representing three unique sequences (designated *A, B*, and *C*) were found in the amplified 446 nucleotide fragment of exon 16 of ovine *RASGRP1*. These sequences were deposited into GenBank with accession numbers MF925341 – MF925343. These variants were the result of three nucleotide substitutions that did not result in any amino acid change. Table 1 shows the overall variant frequencies across all the sheep in this study along with those in sheep with and without flystrike.

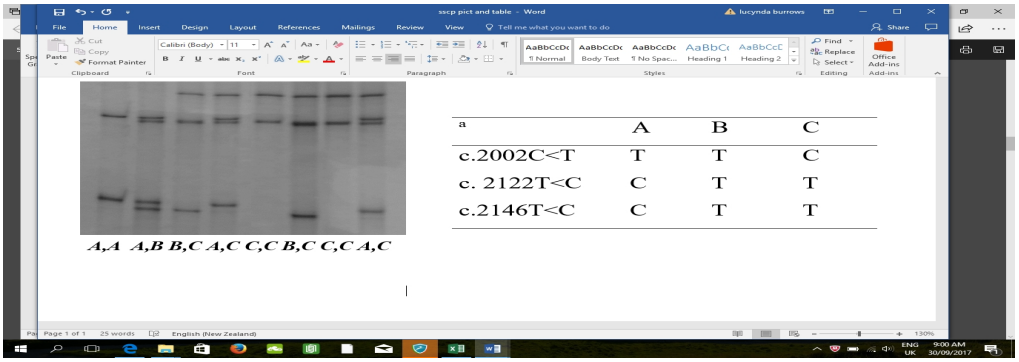

Figure 1: Representative PCR-SSCP patterns for the three variants designated A, B and C of an exon 16 fragment of ovine RASGRP1 shown alongside the single nucleotide polymorphisms associated with them [a].

[a] The numbering of positions follows the guidelines presented on http://varnomen.hgvs.org/recommendations/DNA/variant/substitution/

Table 1: Overall variant frequencies, along with those in sheep with and without flystrike.

***RASGRP1* variant**	***n***	**Frequency**	***n* with fly-struck**	**Frequency in struck sheep**	***n* without flystrike**	**Frequency in unstruck sheep**
A	853	53.9%	468	55.6%	385	52.0%
B	393	24.8%	212	25.2%	181	24.5%
C	336	21.3%	162	19.2%	174	23.5%

The univariate analyses revealed that the presence of *RASGRP1 A* in an animal's genotype was associated with an increased incidence of flystrike (Odds Ratio = 1.404, Pearson chi-square, P = 0.048), whereas *RASGRP1 C* was associated with a decreased incidence of flystrike (Odds Ratio = 0.745, Pearson chi-square, P = 0.049). No association was detected with the presence or absence of *RASGRP1 B* (P = 0.299). The associations observed with *RASGRP1 A* and *C* persisted in the multivariate analyses (Table 2).

Table 2: Binary logistic regression model[1] *for flystrike and variants of ovine RASGRP1, accounting for other factors.*

RASGRP1	**Odds**	**95% Confidence Interval**	**P value**[2]

variant	Ratio	Lower	Upper	
A	1.557	1.091	2.221	0.015
B	1.236	0.914	1.671	0.169
C	0.733	0.539	0.996	0.047

[1]Derived from a multivariate binary logistic regression in which the presence or absence of flystrike was the dependent variable and the independent variable were presence or absence of the gene variant, year, breed, geographical region, and the combined variable of age and gender.
[2]Significant associations are bolded.

Discussion

The aim of this study was to investigate whether variation in exon 16 of ovine *RASGRP1* was associated with the incidence of flystrike. Three variant sequences were identified, two of which (*A* and *C*) were found to be associated the incidence of flystrike. These results are consistent with the genome-wide association study (GWAS) undertaken by Pickering (2013) that suggested that identified *RASGRP1* within 100kbp of the most strongly associated SNP (OAR7_35146905) to flystrike resistance in sheep. Although the role of ovine RASGRP1 in flystrike susceptibility has not been elucidated, it's role in the immune system has been well established in mice and humans. Sheep mount a vast immune response when challenged with flystrike. Neutrophils and eosinophils concentrate on the surface of the skin where strike has occurred, while CD4+, CD8+ and γδ T cells are found in the dermis (Bowles *et al* 1992, Elhay, *et al*. 1994). It is therefore conceivable that nucleotide variation in *RASGRP1* such as that described in this study, may be associated with variation in the expression or function of RASGRP1 thus impacting on immune system function which may potentially play a role in flystrike susceptibility. This would be better tested by looking at the gene expression of sheep with active flystrike.

In mice, it has been found that an overexpression of RASGRP1 in thymocytes results in an increased number of CD8+ T cells (Norment, 2003). In contrast RASGRP1 null (*RASGRP1*$^{-/-}$) mice have a block in thymocyte development and a diminished number of CD4+ and CD8+ T cells (Dower et al., 2000). Thymocytes from *RASGRP1*$^{-/-}$ mice lack TCR- and DAG-induced activation of RAS-ERK signalling (Dower *et al*., 2000). Young *RASGRP1*$^{-/-}$ mice display a defect in positive selection and diminished ERK phosphorylation in the thymocytes (Dower *et al*., 2000). Mice deficient in RasGRP1 therefore have diminished T cell responses and delayed pathogen clearance, this response being functionally similar to the 'exhausted' memory T cell response found during chronic infections (Priatel et al., 2007). A genetic deletion of 200 C-terminal amino acids in mice, also leads to diminished T cell development, although not as severe as what is seen in *RASGRP1*$^{-/-}$ mice (Fuller *et al*., 2012). Such findings emphasise the importance of RASGRP1 in thymocyte selection, and T cell development and function. In sheep RASGRP1 may, therefore, play a pivotal role in regulating T cell response to both the bacteria associated with fleecerot (which predisposes sheep to flystrike) and the invading larvae that results from being flystruck.

In conclusion, variation in the ovine *RASGRP1* could potentially be exploited as a gene marker for use in breeding programmes to reduce the incidence of flystrike thereby improving animal welfare and decreasing the on-farm use of chemicals. Further research examining the extent of variation in ovine *RASGRP1* and the association of the variation with the incidence of flystrike is therefore warranted.

List of References

Bowles, V. M., Grey, S., Brandon, M. R, 1992. Cellular immune responses in the skin of sheep infected with larvae of *Lucilia cuprina*, the sheep blowfly. *Veterinary Parasitology* 44, 151-152.

Bowles, V. M., Chandler, M. K., Verhagen, A., Nash, A. D., Brandon, M. R, 1994. The immune response of sheep infected with larvae of the sheep blowfly Lucilia cuprina monitored via efferent lymph. *Veterinary Immunology and Immunopathology* 40, 341 -352.

Byun, S.O., Fang, Q., Zhou, H., Hickford, J.G.H, 2009. An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry* 385, 174-175.

Dower, N.A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., Stone, J. C, 2000. RasGRP is essential for mouse thymocyte differentiation and TCR signalling. *Nature Immunology* 1, 317-321.

Ebinu, J. O., Stang, S. L., Teixeira, C., Bottorff, D. A., Hooton, J., Blumberg, P. M., Barry, M., Bleakley, C. R., Ostergarrd, H. L., Stone, J. C, 2000. RasGRP links T- cell receptor signaling to Ras. *Blood* 95, 3199-3203.

Elhay, J. M., Hanrahan, C. F., Bowles, V. M., Seow, H. F., Andrews, E. A., Nash, A. D, 1994. Cytokine mRNA expression in skin in response to ectoparasite infection. *Parasite Immunology* 16, 451-461.

Fuller, D. M., Zhu, M., Song, X., Ou-Yang, C.-W., Sullivan, S. A., Stone, J. C., & Zhang, W, 2012. Regulation of RasGRP1 function in T cell development and activation by its unique tail domain. *PloS One* 7, e38796.

Liu, Y., Zhu, M., Nishida, K., Hirano, T., Zhang, W, 2007. An essential role for RasGRP1 in mast cell function and IgE-mediated allergic response. *Journal of Experimental Medicine* 204, 93-103.

Lee, S. H., Yun, S., Lee, J., Kim, M. J., Piao, Z-H., Jeong, M., Chung, J. W., Kim, T-D., Yoon, S. R., Greenberg, P. D., Choi, I. (2009). RasGRP1 is Required for Human NK Cell Function. *The Journal of Immunology* 183, 7931 – 7938.

Ludemann, C., Amer, P., Sise, J, 2010. Value propositions and trait prioritisation of the New Zealand sheep industry, AbacusBio Limited, Dunedin, New Zealand.

Norment, A. M.., Bogatzki, L. Y., Klinger, M., Ojala, E. W., Bevan, M. J., Kay, R. J, 2003. Transgenic Expression of RasGRP1 Induces the Maturation of Double-Negative Thymocytes and Enhances the Production of CD8 Single-Positive Thymocytes. *Journal of Immunology* 170, 1141 -1149.

Pickering, N. K, 2013. Genetics of flystrike, dagginess and associated traits in New Zealand dual-purpose sheep: a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Animal Science at Massey University, Palmerston North, New Zealand.

Priatel, J.J., X Chen, L, A. Zenewicz., H. Shen., K.W., Harder., M.S. Horwitz., H.-S. Teh, 2007. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. *Journal of Immunology* 179, 2143-2152.

Priatel, J. J., Teh, S.-J., Dower, N. A., Stone, J. C., Teh, H.-S, 2002. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity* 17, 617-627.

Raadsma, H. W, 1991. Fleece rot and body strike in Merino sheep. V. Heritability of liability to body strike in weaner sheep under flywave condition. *Crop and Pasture Science* 42, 279-293.

Scobie, D., O'Connell, D, 2010. Breech bareness reduces flystrike in New Zealand crossbred

sheep. *Animal Production Science* 50, 599-602.
Yasuda, S., Stevens, R. L., Terada, T., Takeda, M., Hashimoto, T., Fukae, J., Hortia, T., Kataoka, H., Atsumi, T., Koike, T, 2007. Defective Expression of Ras Guanyl Nucleotide-Releasing Protein 1 in a Subset of Patients with Systemic Lupus Erythematosus. *Journal of Immunology* 179, 4890-4900.
Zhou, H., Hickford, J. G. H., Fang, Q, 2006. A two-step procedure for extracting genomic DNA from dried blood spots on filter paper for polymerase chain reaction amplification. *Analytical Biochemistry* 354, 159-161.