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SHORT COMMUNICATION

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Genome-wide association study to identify genetic loci associated with gastrointestinal nematode resistance in Katahdin sheep

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Summary

Resistance to gastrointestinal nematodes has previously been shown to be a moderately heritable trait in some breeds of sheep, but the mechanisms of resistance are not well understood. Selection for resistance currently relies upon faecal egg counts (FEC), blood packed cell volumes and FAMACHA visual indicator scores of anaemia. Identifying genomic markers associated with disease resistance would potentially improve the selection process and provide a more reliable means of classifying and understanding the biology behind resistant and susceptible sheep. A GWAS was conducted to identify possible genetic loci associated with resistance to *Haemonchus contortus* in Katahdin sheep. Forty animals were selected from the top and bottom 10% of estimated breeding values for FEC from a total pool of 641 sires and ram lambs. Samples were genotyped using Applied Biosystems™ Axiom™ Ovine Genotyping Array (50K) consisting of 51 572 SNPs. Following quality control, 46 268 SNPs were included in subsequent analyses. Analyses were conducted using a linear regression model in PLINK v1.90 and a single-locus mixed model in SNP AND VARIATION SUITE. Genome-wide significance was determined by a Bonferroni correction for multiple testing. Using linear regression, loci on chromosomes 2, 3, 16, 23 and 24 were significantly associated at the genome level with FEC estimated breeding values, and we identified a region on chromosome 2 that was significant using both statistical analyses. We suggest a potential role for the gene *DIS3L2* for gastrointestinal nematode resistance in Katahdin sheep, although further research is needed to validate these findings.

Keywords GWAS, parasite resistance, sheep

Of the gastrointestinal nematodes (GIN) that infect small ruminants, the abomasal parasite *Haemonchus contortus* arguably represents the greatest economic concern. *H. contortus* possesses the highest prevalence of anthelmintic resistance and is the most abundant GIN (Fleming *et al.* 2006). Female worms can produce 5000–15 000 eggs daily, resulting in rapid accumulation of infective larvae on pastures (Emery *et al.* 2016). Fourth-stage larvae and adult nematodes consume blood through the hosts' abomasal mucosa (Emery *et al.* 2016). Individual worms can remove up to 30 µl of blood per day, which can cause fatal anaemia in young or immunocompromised animals (Zajac 2006; Emery *et al.* 2016). *H. contortus* infections account for significant production losses, and concerns regarding treatment costs and anthelmintic resistance have encouraged the development of other methods of GIN control (Saddiqi *et al.* 2011). Current selection strategies use phenotypic markers such as faecal egg count (FEC), antibody assays, packed cell volume (PCV) and FAMACHA visual anaemia score to identify animals that are more resistant to *H. contortus* (Burke & Miller, 2008; Shaw *et al.* 2012; Aguerre *et al.* 2018). Phenotypic selection can be labour intensive and costly, and accuracy of selection depends upon many factors that may be difficult to control, such as variations in natural helminth infection and environmental load depending upon the season (Woolaston & Baker, 1996; Uriarte *et al.* 2003; Jackson & Miller, 2006).

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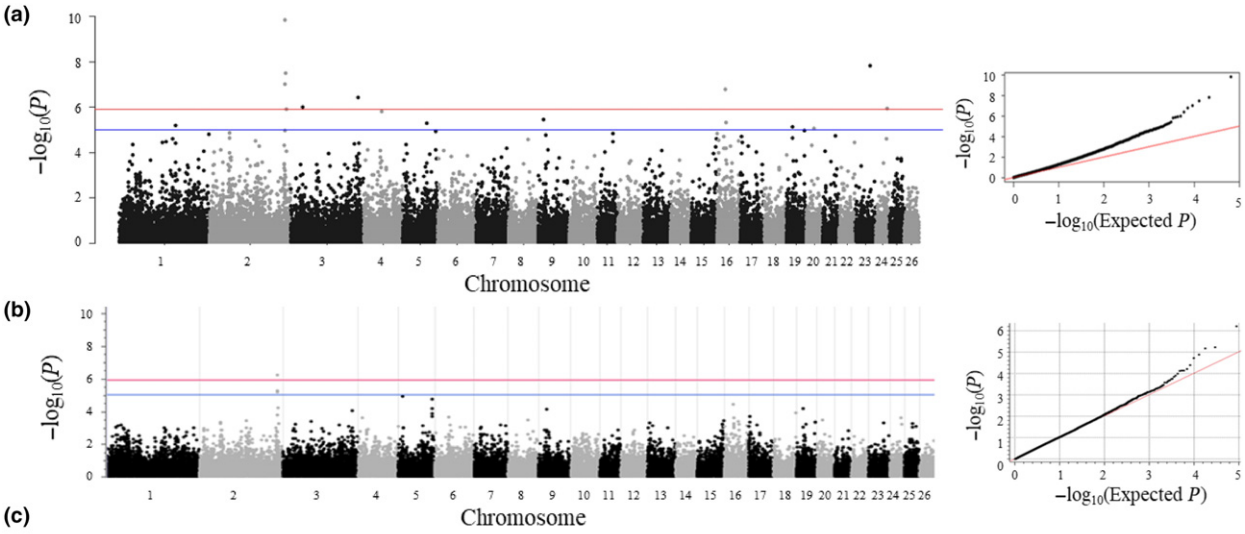

(c)

dbSNP ID	Chromosome	Position (bp)	Unadjusted *P*-Value		MAF: Susceptible	MAF: Resistant	Closest Gene (within 10 kbp of marker)
			LR	SLMM			
rs429321847	1	172,756,008	6.60E-06		0.594	0.229	
rs410136357	2	232,517,234	9.96E-08		0.156	0.565	852 bp 3′ of *PDE6D*
rs406850490	2	232,694,986	1.48E-10	5.98E-07	0.094	0.479	2,507 bp 5′ of *DIS3L2*
rs422243920	2	232,725,251	3.15E-08	5.82E-06	0.719	0.208	*DIS3L2*
rs405054059	2	236,083,199	1.25E-06		0.656	0.188	
rs427558829	3	35,981,310	1.01E-06		0.656	0.250	*ALK*
rs407346502	3	206,105,241*	3.82E-07		0.594	0.146	*C3AR1*
rs419002101	4	55,932,372⁺	1.51E-06		0.438	0.146	
rs55632043	5	73,009,961	5.23E-06		0.188	0.646	
--	9	16,789,975⁺	3.58E-06		0.594	0.146	
rs426380155	16	25,937,201#	1.61E-07		0.375	0.021	
rs424643260	16	28,117,107	4.85E-06		0.469	0.104	
rs406978752	19	18,983,777	7.68E-06		0.281	0.583	*GRM7*
rs410079568	20	23,317,975#	8.90E-06		0.188	0.500	
rs399876637	23	45,403,050⁺	1.43E-08		0.469	0.083	*SLC14A2*
rs423186265	24	34,323,040	1.17E-06		0.313	0.000	6,587 bp 5′ of *ZP3*

Figure 1 Significant SNPs identified through linear regression and single-locus mixed-model GWASs. Genome-wide significance is defined by a Bonferroni correction for multiple testing [$P \leq -\log_{10}(1.25 \times 10^{-6})$; red line] and genome-wide suggestive is defined by [$P \leq -\log_{10}(1 \times 10^{-5})$; blue line]. (a) Left: Manhattan plot for the linear regression model; right: quantile–quantile plot for the linear regression model. (The QQ plot shows an early departure of observed from expected *P*-values, suggesting that this method does not sufficiently account for *P*-value inflation and may indicate the presence of type I error.) (b) Left: Manhattan plot for the single locus mixed-model; right: quantile–quantile plot for the single locus mixed-model. (c) Table displaying results for both models. LR, Linear regression; SLMM, single-locus mixed-model; MAF, minor allele frequency. Markers identified in previous studies are denoted as: *Periasamy et al. (2014), ⁺Atlija et al. (2016), #Sallé et al. (2012).

It is hypothesised that inherited GIN resistance is polygenic and related to the immune system (Saddiqi *et al.* 2011; Atlija *et al.* 2016; Aguerre *et al.* 2018). Protection against GIN has been associated with the T helper 2 (Th2) immune response (Moncada *et al.* 2003) characterised by secretion of interleukin-10 (IL-10) and other cytokines which promote recruitment of eosinophils, basophils and mast cells to control infection and mediate helminth expulsion (Begley & Nicola, 1999; Hussaarts *et al.* 2014; McRae *et al.* 2015). The Th2 response has also been described as a mediator for acute wound healing during helminth infection (Chen *et al.* 2012). IL-10 expression promotes maintenance of the Th2 response by inhibiting the development of Th1 cells and thereby preventing the expression of proinflammatory cytokines such as interferon gamma (IFN-γ) (Bigley & Nicola 1999; Couper *et al.* 2008).

Katahdin sheep are an economically important breed in the United States, ranking within the top six of registered breeds for the last several years (Morgan 2016). The Katahdin breed was developed through crosses of British wool breeds with the St Croix hair sheep (Wildeus 1997). The St Croix breed has been noted for its immune response

and relative resistance to GIN (Burke & Miller 2004; Bowdridge *et al.* 2015). Some mature Katahdin ewes have been shown to have GIN resistance traits similar to St Croix upon natural infection (Burke & Miller 2002). Recent work estimated the heritability (h^2) of FEC in Katahdin lambs at 60 ($h^2 = 0.18–0.26$) and 120 ($h^2 = 0.23–0.46$) days of age (Ngere *et al.* 2018), and periparturient ewes at lambing ($h^2 = 0.29–0.41$) and 30 days postpartum ($h^2 = 0.17–0.31$) (Notter *et al.* 2018). These generally moderate heritabilities for FEC suggest that parasite resistance can be improved through genetic selection.

In the current study animals were selected from a pool of 641 Katahdin ram lambs and sires enrolled in the National Sheep Improvement Program database. Selection was based upon high and low EBV for weaning FEC. Katahdin breeders collected stool samples from each animal's rectum and FEC was quantified by a certified parasitology laboratory (LSU & V Tech Vet School). FEC data were submitted to the National Sheep Improvement Program for EBV prediction by LAMBPLAN (MLA 2004) to reduce variability owing to non-genetic factors (Brown & Tier 2003; Ferguson 2016). To generate a more diverse sample set for genotyping, less related animals within each EBV category were chosen using a measure of genetic relatedness based on pedigree (Lewis *et al.* 2005; Kuehn *et al.* 2008) (Fig. S1). Using a multivariate approach, more related individuals were clustered. Individuals from discrete clusters were then selected. In total, 33 ram lambs and seven sires from eight different US farms were selected for inclusion in the GWAS, with 16 of these animals possessing high FEC EBV and 24 animals possessing low FEC EBV. The two categories captured approximately the top and bottom 10% of animals based on FEC EBV, a phenotypic indicator of GIN resistance.

DNA was extracted from blood samples using the phenol–chloroform method as previously described (Sambrook *et al.* 1989). The Applied Biosystems™ Axiom™ Ovine Genotyping Array (50K), which included 51 572 SNPs, was used for genotyping against the OAR v4.0 reference genome assembly. Quality control was performed in PLINK v1.07, first excluding non-autosomal SNPs and SNPs with a call rate less than 0.90, and then a MAF less than 0.01. Following quality control, 4885 SNPs were identified as duplicate markers. These duplicate SNPs were filtered by Fisher's linear discriminate (FLD) genotype cluster quality score (Johnson & Wichern 2002). The marker with the best FLD genotype cluster score for each of the duplicated SNPs was retained. Following quality control and FLD filtering, 46 268 SNPs were included in analyses.

Genome-wide association was first conducted for FEC EBV using a linear regression (LR) model using PLINK v1.90 software (Chang *et al.* 2015). The package 'qqman' in R version 3.5.1 was used to create the Manhattan plot (Turner 2014) (Fig. 1a).A second analysis was performed using a single-locus mixed-model (SLMM) through SNP AND VARIATION SUITE™ version 8.7.2 (Golden Helix, Inc., www.goldenhelix.com) (Fig. 1b). The SLMM fits a kinship matrix to correct for cryptic relatedness as a random effect and offers more stringent control of the false discovery rate (Segura *et al.* 2012; Brzyski *et al.* 2017). Genome-wide significance was defined by a Bonferroni correction for multiple testing [$P \leq -\log_{10}(1.25^{-6})$]. Genome-wide suggestive was defined by [$P \leq -\log_{10}(1 \times 10^{-5})$] (Fig. 1a,b).

Using LR analysis, loci on chromosomes 2, 3, 16, 23 and 24 were significantly associated at the genome level with FEC EBV (Fig. 1a). Interestingly, a locus on chromosome 3 was within the gene *complement C3a receptor 1 (C3AR1)*

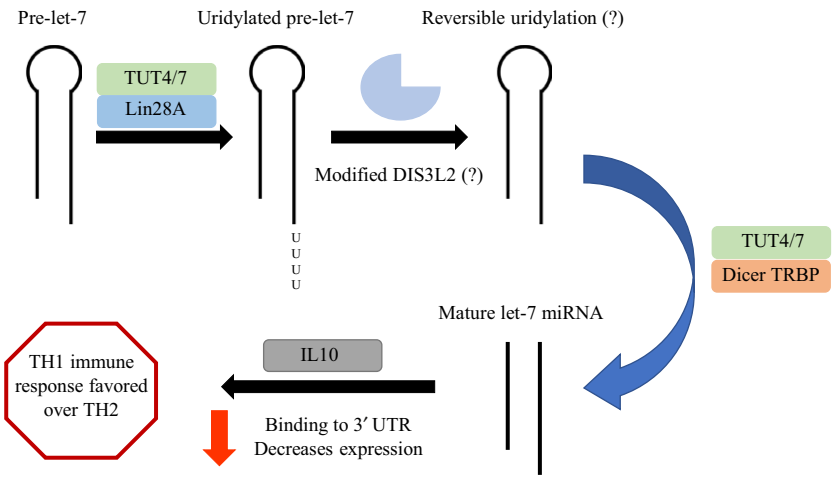

Figure 2 Theorised *DIS3L2* and *let-7* pathway mediating gastrointestinal nematode (GIN) resistance. In the presence of LIN28A and TUT4/TUT7 the pre-*let-7* miRNA is uridylated and marked for degradation by *DIS3L2*. We propose that SNPs associated with *DIS3L2* alter its function such that uridylation of pre-*let-7* becomes a reversible event. *DIS3L2* removes the oligoU tail in this model to allow pre-*let-7* to continue into the maturation pathway with TUT4/TUT7 and Dicer. Mature *let-7* miRNAs bind to the IL10 3′UTR and prevent/reduce IL-10 expression, ultimately allowing the Th1 immune response to be favored over the Th2, resulting in decreased resistance to GIN infection.

and the locus on chromosome 16 was 87 kb upstream of the gene *integrin subunit alpha 2 (ITGA2)*. *C3AR1* has been reported to be differentially expressed in more susceptible vs resistant sheep (Ahmed *et al.* 2015) and has been associated with the Th1 immune response (Ghannam *et al.* 2014). Integrin subunit alpha genes have been found to be upregulated in more resistant animals under GIN infection (Zhang *et al.* 2019). The most significant SNP was located 2507 bp upstream of the gene *DIS3 like 3′–5′ exoribonuclease 2* (*DIS3L2*). The MAF of this SNP was over-represented in resistant (0.479) in comparison with susceptible (0.094) sheep (Fig. 1c). An additional SNP was located within the second intron of *DIS3L2*; this SNP reached significance in the LR model and was most significant using the SLMM model (Fig. 1b). Conversely, the MAF of this SNP was over-represented in susceptible (0.719) in comparison with resistant (0.208) sheep (Fig. 1c).

DIS3L2 is the cytoplasmic exoribonuclease required for the decay of uridylated pre-*let-7* and repression of *let-7* (lethal-7) miRNAs in the Lin28A pathway (Chang *et al.* 2013; Ustianenko *et al.* 2013). *Let-7* has been associated with the immune response to parasite infection through the direct regulation of toll-like receptor 4 expression, and researchers found that *in vitro* suppression of *let-7*i miRNAs in human cholangiocyte cells corresponded with decreased *Cryptosporidium parvum* parasite burden (Chen *et al.* 2007). *Let-7* directly affects IL-10 expression through binding to the IL10 3′UTR; both *let-7*a and *let-7*d family members have been associated with repression of IL-10 (Schulte *et al.* 2011; Swaminathan *et al.* 2012). An absence of IL-10 has been shown to result in increased levels of IFN-γ and delayed expulsion of *Trichinella spiralis* in mice (Helmby & Grencis, 2003). Upregulation of IFN-γ has been described in the abomasum and abomasal lymph nodes of sheep that were classified as susceptible to *H. contortus* infection (Zaros *et al.* 2014). Reduction of IFN-γ expression may enhance the immune response to GIN, as this would favour the Th2 cell subset and antibody-associated immune mechanisms (Coltman *et al.* 2001).

This preliminary study suggests that the gene *DIS3L2* may have a role in GIN resistance in Katahdin sheep. We theorise that polymorphisms within or associated with *DIS3L2* moderate its function to allow preferential degradation of the oligoU tail but not degradation of pre-*let-7* itself. Instead, pre-*let-7* may enter the maturation pathway following removal of the oligoU tail (Fig. 2). Considering the small sample size and uncorrected inflation factor in the LR model ($\lambda = 1.51$) (Fig. 1a), additional work is needed to validate these preliminary findings. Validation may be accomplished through increasing the sample size or expanding the study to include other populations of hair sheep. Future research is required to understand the immune mechanisms that differentiate GIN resistance from susceptibility.

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Conflict of interest

The authors have no conflict of interest to declare.

Data availability

QTL and phenotype data are available through the SheepQTLdb of the National Animal Genome Research Program, and can be accessed at https://www.animalgenome.org/QTLdb/supp/?t=FbNc7B5WsJ.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Eigenvector plot showing animal relatedness and phenotypic distribution.