doi:10.1111/j.1365-2052.2006.01557.x

Cloning, mapping and association studies of the ovine *ABCG2* gene with facial eczema disease in sheep

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Summary

Facial eczema (FE) is a hepatogenous mycotoxicosis in sheep caused by the fungal toxin sporidesmin. Resistance to FE is a multigenic trait. To identify QTL associated with this trait, a scan of ovine chromosomes was implemented. In addition, *ABCG2* was investigated as a possible positional candidate gene because of its sequence homology to the yeast PDR5 protein and its functional role as a xenobiotic transporter. The sequence of ovine *ABCG2* cDNA was obtained from liver mRNA by RT-PCR and 5′ and 3′ RACE. The predicted protein sequence shares >80% identity with other mammalian ABCG2 proteins. SNPs were identified within exon 6, exon 9 and intron 4. The intron 4 SNP was used to map *ABCG2* to ovine chromosome 6 (OAR6), about 2 cM distal to microsatellite marker *OarAE101*. Interestingly, this chromosomal region contains weak evidence for a FE QTL detected in a previous genome-scan experiment. To further investigate the association of *ABCG2* with FE, allele frequencies for the three SNPs plus three neighbouring microsatellite markers were tested for differences in sheep selected for and against FE. Significant differences were detected in the allele frequencies of the intronic SNP marker among the resistant, susceptible and control lines. No difference in the levels of *ABCG2* expression between the resistant and susceptible animals was detected by Northern hybridisation of liver RNA samples. However, significantly higher expression was observed in sporidesmin-dosed sheep compared with naïve animals. Our inference is that the *ABCG2* gene may play a minor role in FE sensitivity in sheep, at least within these selection lines.

Keywords ABC transporter, liver, mycotoxin, photosensitivity, quantitative trait locus.

Introduction

Facial eczema (FE) disease is a hepato-mycotoxicosis of ruminants caused by sporidesmin, which is a toxin produced by the saprophytic fungus *Pithomyces chartarum*. This toxin causes severe necrotising inflammation of both the liver and bile ducts in FE-susceptible animals. Secondary photosensitisation occurs due to the inability of the affected animal to excrete phylloerythrin, a photoreactive breakdown product of chlorophyll. As a consequence, phylloerythrin accumulates in the blood leading to localised cellular necrosis when activated by UV-light at exposed skin surfaces (reviewed by Mortimer & Ronaldson 1983).

There is a significant genetic component in resistance to FE, with an estimated heritability of 0.42 (Campbell *et al.* 1981). Several groups of genes have been examined for their involvement in conferring resistance to sporidesmin (Phua *et al.* 1999; Hohenboken *et al.* 2004). One gene family of particular interest is the ABC (ATP-binding cassette) transporter superfamily, which has 48 members in humans and are divided into eight sub-families (A–H; reviewed by Dean *et al.* 2001). This super-family was first highlighted as a potential group of candidate genes when it was found that levels of *PDR5* (*pleiotropic drug resistance protein* 5, *STS1)* expression modulated the sporidesmin sensitivity of *Saccharomyces cerevisiae* (Bissinger & Kuchler 1994). At that time the closest-known mammalian ortholog to the yeast PDR5 protein was ABCB1 (P-glycoprotein, MDR1), as they shared significant overlap in their substrate-specificity profile (Balzi *et al.* 1994; Bissinger &

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Accepted for publication 17 December 2006

Kuchler 1994). Subsequent studies of ovine *ABCB1* ruled out its possible involvement in FE (Longley *et al.* 1999; Morris *et al.* 2004).

Identification of a closer mammalian ortholog of the yeast PDR5 protein is now possible because of the availability of complete sequences of several genomes (Ponting 2001). Our phylogenetic analyses identified the mammalian ABCG subfamily as the most likely homologs of the yeast PDR5 protein, which agrees with published data (Sheps *et al.* 2004). The ABCG family has five members in most mammals, except rodents which have six (Mickley *et al.* 2001). Four function in cholesterol and lipid homeostasis (reviewed by Schmitz *et al.* 2001), whereas ABCG2 is known to function as a xenobiotic transporter in both humans (reviewed by Staud & Pavek 2005) and cattle (Jonker *et al.* 2005). Additionally, mice deficient in ABCG2 were sensitive to diet-dependent phototoxicity (Jonker *et al.* 2002), a clinical phenotype similar to FE. We report here the characterisation of the ovine *ABCG2* gene and its possible involvement in FE in sheep.

Materials and methods

Detection of quantitative trait loci in outcross families

Four outcross families were generated as described in Phua *et al.* (1999). Briefly, four F1 Romney rams were produced from reciprocal crosses of FE resistant and susceptible selection-line animals and mated with 130–170 Romney ewes to generate four outcross pedigrees, with 124–167 progeny per family. The severity of liver damage in sporidesmin-dosed progeny was assessed by measuring serum levels of the liver-specific enzymes gamma-glutamyl transferase (GGT) and glutamate dehydrogenase (GDH) 3 weeks post-dosing. Additionally, there was a cohort of very tolerant animals that did not respond to the initial sporidesmin dose (0.13 mg/kg live-weight); these animals were given a second dose of sporidesmin (0.17 mg/kg live-weight) and their GGT levels were measured 3 weeks after dosing. Data from the first initial dosing were designated GGT and GDH, and combined GGT data from the two dosings were designated GGT21.

The QTL analysis using the outcross families was conducted via primary and secondary screens. Ten evenly spaced microsatellite markers on OAR6 that were heterozygous in at least one of the four sires were used. In the primary screen, inheritance of the sire alleles was determined for 22 of the most resistant and 22 of the most susceptible progeny (based on GGT data). If a suggestive or significant QTL was detected, then all progeny ($n = 124$–167/sire) of the heterozygous sires were genotyped and analysed.

The GGT and GDH data together with the genotype information were analysed using the Haley-Knott method (Knott *et al.* 1996). In our original analyses, genome-wide suggestive and significant linkage probabilities were determined to be $P = 0.0016$ and $P = 0.000054$ respectively (Lander & Kruglyak 1995). Subsequently more appropriate genome-wide significance thresholds were set by permutation testing (Churchill & Doerge 1994) using 1000 replicates. These latter values were used in Fig. 1.

Romney facial eczema selection lines

The selection lines were as described previously (Phua *et al.* 1999; Morris *et al.* 2004). Briefly, genetic selection for FE resistance and susceptibility in Romney sheep started in 1975, with the control line introduced in 1982 (Morris *et al.* 1989). The selection response was assessed from

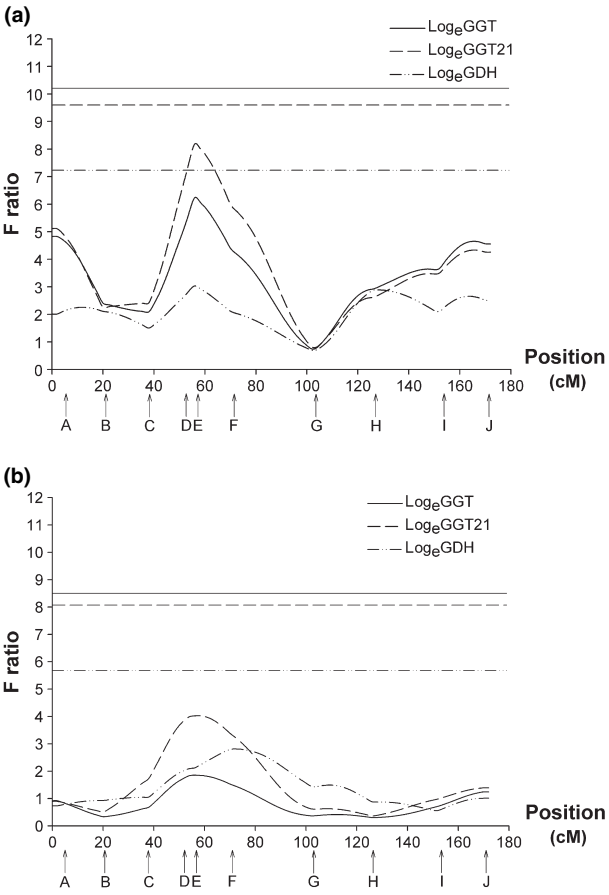

Figure 1 A region detected on OAR6, associated with facial eczema (FE) in outcross pedigrees. Selective genotyping was used in the primary genome screen (a). In the secondary screen (b), all progeny of informative sires were genotyped and analysed. The vertical axis plots the *F*-value for the allele-within-sire term (1 numerator d.f. for each of the four families). The three horizontal lines indicate the genome-wide suggestive levels as determined by permutation analysis for the three FE traits analysed (log$_e$GDH, log$_e$GGT and log$_e$GGT21). The positions of microsatellite markers used in the genome scan are indicated by the arrows below each graph: A = *OarCP125*, B = *BM9058*, C = *MCM53*, D = *BM1329*, E = *OarAE101*, F = *BM143*, G = *BM4621*, H = *BM4311*, I = *OarJMP8* and J = *BL1038*.

changes in the FE breeding value for $\log_e$GGT. When animals were sampled to test for FE differences (birth years 1991–1995), the resistant and susceptible lines differed by a factor of ×3.7 (1991) and ×6.9 (1995) (Morris *et al.* 2004).

Isolation of sheep liver samples

Liver samples as a source for total RNA were obtained from 19 resistant- and 18 susceptible-line animals born in years 1995–1997 and 2000–2002. Naïve animals were undosed. Dosed animals were animals orally challenged with sporidesmin at 0.1 or 0.4 mg/kg live-weight. Sporidesmin-dosed animals used in the Northern hybridisation experiment all received 0.1 mg/kg, with the exception of R8 and R9 (Fig. 2) which received 0.4 mg/kg. Animals were killed 24 h after dosing for collection of livers: liver samples were snap-frozen in liquid nitrogen and stored at −80 °C.

Amplification and sequencing of ovine *ABCG2* cDNA

Primers were designed using VECTOR NTI SUITE 7.1 (InforMax Inc.) based on the human *ABCG2* gene (NM_004827). Primer sequences, melting temperatures and the sizes of the amplified fragments are shown in Table S1. Sequencing was done on cDNA obtained after PCR amplification, and 5′ and 3′ RACE.

Single nucleotide polymorphism (SNP) discovery and genotyping

Human and mouse genome sequences for *ABCG2* were found using the UCSC genome browser (http://genome.ucsc.edu/), and megaBLAST (Zhang *et al.* 2000) was used to extract equivalent bovine genomic sequences. The genome sequences were then aligned against the ovine cDNA sequence using the SPIDEY algorithm (Wheelan *et al.* 2001) set to adjust for divergent sequences. Primers were then designed to flank intron 4 (about 1 kb) of the *ABCG2* gene (Table S1). Approximately 100 ng of genomic DNA from the four AgResearch International Mapping Flock (IMF) sires (Crawford *et al.* 1995) was used in a PCR reaction, and SNPs were detected by sequencing the PCR products.

The intronic SNP marker (ss65824074:G>A) was genotyped in both the full IMF population ($n = 125$) and FE selection-line animals ($n = 176$), including 66 resistant, 66 susceptible and 44 control animals. Two other SNPs (ss65824075:G>A and ss65824134:C>T), discovered in one of the selection-line animals during cDNA sequencing, and three microsatellite markers (*JL36*, *CSAP14E*, *OarAE101*), which were within 2.4 cM of the *ABCG2* locus, were genotyped in the selection-line animals. All three SNPs were genotyped using primer extension with analyte detection in a MALDI-TOF mass spectrometer (Sequenom Inc.) (Tang *et al.* 1999) (Table S1).

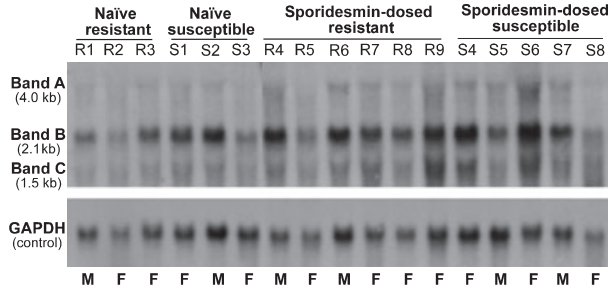

Figure 2 Northern hybridisation showing expression of the *ABCG2* transcripts in livers of resistant and susceptible selection-line animals, both naïve and sporidesmin-dosed. The gender of individual animals is indicated below the lanes (M = male, F = female).

Allele frequencies of markers in FE selection lines

Allele frequencies for each marker were compared between the resistant, susceptible and control selection lines using Chi-squared statistics. The significance level for these statistics was found using the peddrift method (Dodds & McEwan 1997), which accounts for genetic drift, founder and sampling effects. The method finds the distribution of the Chi-squared statistics under the null hypothesis of no selection on the marker by simulating genotypes through the actual pedigree data.

Northern hybridisation

Northern blots containing 20 μg of total liver RNA per animal were hybridised with (α–^{32}P)dCTP labelled probes. The *ABCG2* probe consisted of part of the linker region plus the first five transmembrane spanning domains.

The autoradiographs were scanned using an ImageScanner^{TM} II and LabScan^{TM} (Amersham Biosciences), and quantified using ImageQuant TL^{TM} (Amersham Biosciences). To correct for variation in loading or transfer, the total absorbance of each band was determined and normalised to the total absorbance units for a *GAPDH* control (Charlier *et al.* 2001) in the same sample. Absorbance values were log-transformed to account for the increased variance scaled to the mean and then analysed by least squares (ANOVA) methods. The initial model included the main effects of sporidesmin exposure and genetic lines along with their interaction. The interaction was dropped from subsequent models as it was found to be non-significant.

Results and discussion

Facial eczema quantitative trait loci experiment

As part of a whole genome-scan QTL experiment, 10 microsatellite markers evenly spaced on OAR6 were genotyped in four outcross families in the primary screen. The

analysis revealed weak evidence for a QTL between markers *MCM53* and *BM4621*, with its peak around *OarAE101*. Initial analysis using the method of Lander & Kruglyak (1995) showed significant linkage in this region for $\log_e$GGT21 and a suggestive linkage for $\log_e$GGT. However, subsequent analyses using permutation thresholds (Churchill & Doerge 1994) gave a *F*-ratio for $\log_e$GGT21 nearing the suggestive level (Fig. 1a). In addition, the 95% confidence interval spanned all 10 markers tested. When the four families were considered individually, one family (R × S Sire 3) showed suggestive linkage for $\log_e$GDH, $\log_e$GGT and $\log_e$GGT21 in this region (data not shown).

In the secondary genome screen where all progeny of heterozygous sires were analysed, similar QTL results were obtained with the combined families' data (Fig. 1b), except that the 95% confidence interval for $\log_e$GDH was reduced to the 20–170 cM interval on OAR6. When the four families were analysed individually, the R × S Sire 3 family gave a significant QTL (genome-wide $P < 0.05$) in this same region (between *MCM53* and *BM4621*) for $\log_e$GDH, with an estimated allele substitution effect of 0.84 phenotypic SD and a 95% confidence interval spanning the 44.7–86.7-cM region (data not shown).

Ovine *ABCG2* cDNA sequence

Using primers designed to the human *ABCG2* sequence (Table S1), the full coding region of the ovine *ABCG2* gene was obtained. The ovine sequence showed conserved motif structure, domain organisation and sequence similarity to the human *ABCG2* gene.

Several SNPs have been identified in the human *ABCG2* gene, and three of these SNPs are known to affect the function of the ABCG2 protein. The Q141K mutation affects the transport efficiency of the protein (Mizuarai *et al.* 2004), whereas the S441N mutation is known to alter the localisation of the mature protein from the cell membrane to an intracellular location. However, at the time of our study, the only known mutation that altered ABCG2 protein function was R482X, which was discovered in drug-selected cell lines and altered substrate specificity (Honjo *et al.* 2001). Subsequent studies failed to detect the R482X mutation in human populations (Honjo *et al.* 2002).

The entire coding region of the ovine *ABCG2* gene from two resistant and two susceptible selection-line sheep was sequenced and two synonymous SNPs were detected. The first SNP was a G>A transition at position 554 of the cDNA sequence (exon 6, ss65824075:G>A), and the second SNP was a C>T transition at position 973 (exon 9, ss65824134:C>T). Additionally, the 1059–1800 bp coding region encompassing amino acids 353–600 (exons 9–13), was sequenced from eight resistant- and eight susceptible-line animals, and no polymorphisms were detected. These data suggest that there are no differences in the primary structure of the ABCG2 protein between FE resistant and susceptible selection-line animals.

Linkage mapping of the *ABCG2* locus

To find *ABCG2* SNPs for linkage mapping in the AgResearch IMF animals, intron 4 was sequenced for four IMF sires. Intron 4, which is 813 bp in length (DQ886529), has the classical GT donor and AG acceptor splice boundaries. Six SNPs and a single insertion were discovered within this intron. Three of the four IMF sires were heterozygous for a SNP at position 671 (ss65824074:G>A). The ss65824074:G>A mutation was linked to markers on OAR6 (Fig. S1) with a maximum two-point LOD score of 16.1 with *LSCV43* on the framework map of Maddox *et al.* (2001) and a recombination fraction of 0.01. Multipoint mapping placed *ABCG2* 2 cM distal to *OarAE101*, consistent with that predicted from comparative mapping between human, cattle and sheep. The *ABCG2* locus falls within the peak of a FE QTL on OAR6 (Fig. 1).

Association studies of markers in facial eczema selection lines

Association studies of *ABCG2* and nearby microsatellite markers with FE were carried out in FE selection lines. Only the *ABCG2* SNP ss65824074:G>A showed a significant difference in allele frequencies between the selection lines ($P = 0.044$): the susceptible line (allele A frequency = 0.17) differed from the resistant (A = 0.52) and control (A = 0.58) lines. In addition, haplotypes composed of the three *ABCG2* SNPs showed significant association within FE selection lines ($P = 0.03$). Therefore in addition to *ABCG2* being a positional and functional candidate gene for the OAR6 FE QTL region, this association suggests its possible involvement in FE disease.

Northern analysis of the *ABCG2* gene

Northern hybridisation was carried out to determine if there was any differential expression of *ABCG2* in liver between resistant and susceptible sheep under naïve and sporidesmin-dosed conditions. Three RNA bands were detected (Fig. 2); band B (2.1 kb) was the most predominant transcript with a size consistent with the full-length cDNA sequence (2191 bp). The Northern autoradiograph was scanned and quantified, and the expression of the 2.1 kb *ABCG2* transcript was calculated relative to the *GAPDH* control. Statistical analysis showed no differential expression of the *ABCG2* gene between resistant and susceptible sheep, in both naïve and sporidesmin-dosed animals. No difference in expression was detected between male and female sheep despite a number of reports concerning gender differences in *ABCG2* expression (Krishnamurthy & Schuetz 2005).

Interestingly, there was a 1.56-fold increase of *ABCG2* expression following exposure of animals to sporidesmin when compared with naïve animals ($P = 0.017$). As expression of *ABCG2* in stem cells is known to be modulated by the transcription factor HIF-1 (hypoxia-inducible factor 1) (Krishnamurthy *et al.* 2004) and HIF-1 is also expressed in liver, it is possible that the observed increase in *ABCG2* transcription may be via HIF-1 activation in response to a cytotoxic hypoxic state induced by sporidesmin. Moreover, sporidesmin-mediated induction of *ABCG2* may enable it to act as a phase III defence mechanism by accelerating removal of the xenobiotic via enhanced excretion into the bile (reviewed by Krishnamurthy & Schuetz 2005).

Although only liver expression was studied, *ABCG2* is known to be expressed in other tissues and its expression or function in those tissues may be of more relevance to FE. For instance, in mice the ABCG2 protein modulates both hepatobiliary excretion and intestinal absorption of substrates (reviewed by Staud & Pavek 2005). Therefore, it would be of interest to study *ABCG2* in the intestines of FE sheep.

Acknowledgements

The authors thank K. Paterson for designing the Mass Extend assays, A. Beattie and T. Van Stijn for CRI-MAP analysis, B. Auvray for haplotype estimation and Dr R. McLaren and Dr O. Keane for providing the GAPDH clone. E. J. Duncan was supported by a MeatNZ grant to S. H. Phua.

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Supplementary Material

The following supplementary material is available for this article online from http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01557.x

Figure S1 Localisation of the *ABCG2* locus on the sex-averaged map of ovine chromosome 6 (OAR6).

Table S1 PCR primer sequences for ovine *ABCG2*.

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