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ASSOCIATION BETWEEN MYOSTATIN DNA MARKERS AND MUSCULARITY IN ANGUS CATTLE

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SUMMARY

Myostatin (MSTN) is a potent negative regulator of skeletal muscle development. High genetic variability has been observed in *bovine MSTN* which includes 6 specific disruptive mutations responsible for extreme muscular hypertrophy in cattle. In this study the effect of non-disruptive *MSTN* polymorphisms on muscularity was examined in a population of 594 Angus cattle. Six tag SNP (single nucleotide polymorphism), which included 5 non-disruptive SNP and the disruptive 821 del11 mutation, were genotyped in each animal and haplotype phase was inferred. Eleven haplotypes were found in the Angus population and the muscular hypertrophy marker (821del11) was confined to 2 haplotypes (8 and 11). Association between *MSTN* haplotypes and eye muscle area (EMA) at weaning age was tested using multiple linear regression. In the regression analysis comprising all cattle, haplotypes 1, 2, 4, 7 and 9 had significant regression coefficients ($P<0.05$) relative to haplotype 5. These haplotype associations were confirmed in a second analysis that contained only cattle without the 821del11 muscular hypertrophy marker ($n=528$). These results indicate that other *MSTN* DNA markers, when assessed as haplotypes, are associated with variation in EMA and therefore contribute to differences in muscularity.

INTRODUCTION

Myostatin (MSTN), a secreted protein, is a member of the transforming growth factor – β superfamily. Loss of MSTN function causes large increases in muscle mass and hence, MSTN is regarded as a potent negative regulator of skeletal muscle mass (McPherron *et al.* 1997). This large increase in muscle mass is termed muscular hypertrophy or double muscling. Naturally occurring mutations in the *MSTN* gene that are implicated in double muscling have been reported in cattle (Grobet *et al.* 1998), sheep (Clop *et al.* 2006), humans (Schuelke *et al.* 2004) and dogs (Mosher *et al.* 2007).

Analysis of the bovine *MSTN* sequence prompted by the genetically heterogeneous nature of double muscling, has also uncovered a series of non-disruptive polymorphisms (Grobet *et al.* 1998; Crisa *et al.* 2003; Dunner *et al.* 2003; O'Rourke *et al.* 2009). These studies show considerable genetic diversity within *MSTN*, which may also contribute to variation in muscle mass.

In a previous study, 18 *MSTN* DNA markers, including the 821del11 double muscling marker, were identified in a sub-group of Angus cattle (O'Rourke *et al.* 2007). The purpose of this study was to examine the effects of non-disruptive *MSTN* polymorphisms on muscularity in a larger population of Angus cattle. We tested the null hypothesis that only the double muscling marker 821del11 was contributing to variation in muscularity.

MATERIALS AND METHODS

Data and DNA samples for 594 Angus cattle born between 1998 and 2006 from a NSW Department of Primary Industries Research Herd at Glen Innes, Australia were used in this study. This herd was established for research purposes in 1988 and comprised high and low muscle selection lines. Selection was based on muscle score (McKiernan, 1990) as assessed at weaning. Of the 594 cattle used, 324 (198 female and 126 male) were classified as high muscle and 270 (154 female and 116 male) were from the low muscle line. Measurements for eye muscle area (EMA) were taken at weaning age (approximately 9 months of age) by experienced and/or accredited technicians using real-time ultrasound 3.5 MHz/180-mm linear array animal science probe (Esoate Pie Medical, Maastricht, Netherlands).

A tag SNP genotyping approach was employed to determine genotypes at 6 *MSTN* polymorphic sites. The tag SNP included 5 non-disruptive polymorphisms (2 promoter polymorphisms, 1 in intron 1, and 2 in the 3' untranslated region) that did not alter the length of the *MSTN* coding region and 1 disruptive mutation (821 del11) in Exon 3; a frameshift mutation, which introduces a premature stop codon. All animals with the 821del11 mutation were heterozygous at this site; no 821 del11 homozygotes were included in the study. A polymerase chain reaction/restricted fragment length polymorphism method was used for genotyping the promoter and 3' untranslated region polymorphisms and a primer extension methodology was used for the intron 1 polymorphism. Genotypes at the 821 del11 site were determined by real time PCR (O'Rourke *et al.* 2009). At each polymorphic site the allele differing from the GenBank reference database sequences AF320998 and AF348479 was designated as the mutated allele.

Haplotype phase was inferred from the genotypic data using PHASE v2.1.1 (Stephens et al. 2001; Stephens and Scheet 2005). Ambiguous genotypes were also inferred using PHASE v2.1.1. The association between each haplotype and eye muscle area (EMA) at weaning age was tested using multiple linear regression (SAS 9.1.3; SAS Institute). The statistical model accounted for the main effects of sex, muscling selection line and birth year. Interaction effects were not fitted due to low sub-class observations. Sire was included as a random term and weight at the time of measurement was used as a covariate. Haplotype 5, which does not contain the disruptive 821del11 mutation was used as the reference haplotype (regression coefficient = 0) in the analysis. The association analysis initially included all animals in the cattle population that had either 0 (n=528) or 1 (n=66) copy of the 821 del11 mutation. A second analysis was performed which excluded the 821 del11 heterozygotes.

RESULTS AND DISCUSSION

In this study an Angus cattle population was genotyped at 6 *MSTN* polymorphic sites, which included the 821 del11 disruptive mutation in Exon 3, historically associated with double muscling in Belgian Blue cattle (Grobet *et al.* 1997). The 6 sites were selected using a tag SNP approach from a total of 18 *MSTN* sites previously found in a sub-group of this cattle population (O'Rourke *et al*. 2007). Haplotype phase was inferred for each animal and 11 haplotypes were identified (Table 1). The disruptive 821 del11 mutation was confined to haplotypes 8 and 11. The low frequency of haplotype 8 prompted confirmation of genotypes for this animal. Parent genotypes for the 821del11 mutation indicated that the mutation had been inherited from the sire (heterozygous for haplotype 11) and parentage was confirmed by DNA testing. We have therefore deduced that haplotype 8 has arisen from recombination of the paternal gamete indicating that haplotype 11 is the ancestral double muscling haplotype in this cattle population. Haplotype 10, also in low frequency, was confirmed as the most likely allele combination by pedigree analysis. Moderate to high frequency was observed for the other haplotypes with haplotype 7 the most prevalent.

Table 1. Haplotype diversity in the *myostatin* gene for 594 Angus cattle

Haplotype	***N***	tag SNP					
		1	2	3	4	5	6
1	162			+			
2	57						+
3	75					+	
4	136	+		+			
5	68	+					+
6	32	+				+	+
7	555	+				+	
8	1	+			+		
9	35	+	+				+
10	2	+	+			+	
11	65	+	+		+		

+ indicates the presence of the mutant allele with respect to the reference sequences AF320998 and AF348479. Tag SNP 1 and 2 are the promoter polymorphisms, site 3 is in intron 1, site 4 is the 821 del11 mutation in exon 3, and 5 and 6 are in the 3' untranslated region. *N* = haplotype observations (2/animal)

The association between *MSTN* haplotype and ultrasound measurements for eye muscle area was determined by multiple linear regression (Table 2). The alleles were assessed collectively as haplotypes offering greater power for association studies, particularly for complex traits where many markers of small effect and few with large effect may be linked to phenotypic variation (Hayes and Goddard 2001). Initially, the association between haplotype and EMA was tested in the entire cattle population. Relative to haplotype 5, all haplotypes except for haplotype 11 had a negative regression coefficient, suggesting haplotype 5 is associated with the second largest EMA. The positive regression coefficient for haplotype 11 and its association with the largest EMA was expected since this group contains all but one of the 821 del1l heterozygotes, and therefore adds a quality control aspect to the analysis. Haplotypes 9, 2, 1, 4 and 7 ranked in order of their regression coefficients (*b*), showed significantly less EMA compared to haplotype 5 ($P<0.05$).

Table 2. Haplotype association with eye muscle area (EMA, cm^2) at weaning age in Angus cattle

Haplotype	All cattle			Cattle without 821 del11 marker		
	N	***b*** ± s.e.	***P***	***N***	***b*** ± s.e.	***P***
1	162	-2.68 ± 0.85	0.0018	154	-3.01 ± 0.89	0.0008
2	57	-3.74 ± 1.37	0.0066	57	-4.13 ± 1.37	0.0027
3	75	-0.63 ± 0.96	0.5131	69	-0.84 ± 1.01	0.4085
4	136	-1.89 ± 0.89	0.0336	133	-2.12 ± 0.92	0.0215
5	68	0	-	64	0	-
6	32	-0.82 ± 1.16	0.4797	31	-0.96 ± 1.20	0.4259
7	555	-1.79 ± 0.76	0.0184	516	-1.81 ± 0.79	0.0227
8	1	-1.05 ± 5.14	0.8386	0	-	-
9	35	-4.14 ± 1.16	0.0004	30	-4.80 ± 1.26	0.0001
10	2	-2.53 ± 3.67	0.4901	2	-2.43 ± 3.73	0.5149
11	65	1.43 ± 1.08	0.1865	0	-	-

[a]All haplotypes are relative to haplotype 5. *N*, number of haplotype observations (2/animal); *b*, EMA regression coefficient.

The double muscled phenotype occurs in animals homozygous for a disruptive *MSTN* mutation. Double muscled cattle can have up to a 20% increase in muscle mass indicating that these disruptive mutations have a large effect on muscularity (Grobet *et al.* 1997). The partially recessive mode of inheritance for these mutations means that in the heterozygous form, significant increases in muscling are also observed (Gill *et al.* 2008; O'Rourke *et al.* 2009). In this study, the initial association analysis included cattle heterozygous for the 821 del11 mutation. To determine if the original haplotype associations were confounded by the inclusion of a double muscling marker, the analysis was repeated in a reduced population which excluded all 821 del11 heterozygotes (haplotypes 8 and 11; Table 2). The results again showed that haplotype 5 was associated with the largest EMA for each the 'functional' haplotypes, and haplotypes 9, 2, 1, 4 and 7 remained significant ($P<0.05$) and their ranking relative to haplotype 5 had not altered.

In conclusion, the results presented in this study indicate that other *MSTN* polymorphisms that have not been implicated in double muscling are associated with variation in muscularity.

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