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Novel SNPs in the *Ankyrin 1* gene and their association with beef quality traits

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ABSTRACT

Single nucleotide polymorphisms (SNPs) in the promoter region of bovine *Ankyrin 1* (*ANK1*) have been associated with tenderness and intramuscular fat level in beef. The objectives of this study were to characterise novel DNA variants in the coding region of bovine *ANK1* and test for association with beef quality traits. A 3 kb region of *ANK1* cDNA was amplified and sequenced in 32 Charolais cattle using five sets of overlapping primers. Eighteen SNPs were identified and a predicted exon was confirmed. An in silico translation indicated that SNP4 and SNP16 were non-conservative. Three SNPs were genotyped in 158 crossbred cattle ($n = 158$) with associated meat quality data. SNP6 was associated with texture scores while SNP17 was associated with juiciness. Haplotype (cHAP) 1 was associated with lightness, redness, ultimate pH, as well as sarcomere length. Alleles of the *ANK1* gene could be potential targets for gene-assisted selection to improve a range of meat quality traits in beef.

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1. Introduction

Meat quality can be characterised experimentally using a number of objectively and subjectively measured parameters (Erkens et al., 2010) and includes sensory traits such as tenderness, juiciness, flavour and colour (Bernard et al., 2007), as well as nutritional value and technological quality (Hocquette, Lehnert, Barendse, Cassar-Malek, & Picard, 2007). There is evidence that the consumer is prepared to pay more for what are deemed to be superior products (Gill, Bishop, McCorquodale, Williams, & Wiener, 2009). While environmental factors such as nutritional strategy, pre-slaughter and *post mortem* processing technologies play a role in influencing meat quality, genetics is also a major contributor to the quality of meat (Erkens et al., 2010). In order to satisfy consumers' preferences and improve consistency in quality of beef, a deeper understanding of the genetic influences on meat quality is necessary (Gao, Zhang, Hu, & Li, 2007). Gene-assisted selection (GAS) allows for the inclusion of genetic information in breeding programmes for economically important traits (Gao et al., 2007), however, to date, only a few single nucleotide polymorphism (SNP) markers (e.g. *CAPN1*, *CAST* etc.) have been incorporated in commercial DNA tests for shear force/tenderness and other key traits in beef cattle (Casas et al., 2006; Costello et al., 2007; Page et al., 2002; Schenkel et al., 2006). Polymorphisms in other genes e.g. *MYPN*, *MHC*, *TTN* have been correlated with water holding capacity, tenderness and marbling but not commercialised (Grzes et al., 2007; Jiao, Zan, Liu, Wang, & Guo, 2010; Yamada et al., 2009). There is thus a gap in knowledge and a need for further research on muscle-related genes that may be relevant for meat quality.

A quantitative trait locus (QTL) affecting meat quality traits is located on bovine chromosome 27 (http://www.animalgenome.org/cgi-bin/QTLdb/BT/index) and *Ankyrin 1* (*ANK1*) is a candidate gene in this region. Ankyrins are a family of structural proteins that have binding sites for a number of integral membrane and cytoskeleton proteins (Gallagher, Tse, Scarpa, Lux, & Forget, 1997; Moon & Lazarides, 1984; Nelson & Lazarides, 1984; Weaver, Pasternack, & Marchesi, 1984) and attachment of the cytoskeleton to the plasma membrane is mediated via ankyrin interactions (Rubtsov & Lopina, 2000). The regulatory domain of the ankyrin 1 protein is sensitive to proteolysis (Rubtsov & Lopina, 2000). Proteolysis of key myofibrillar and cytoskeletal proteins causes breaking of linkages between myofibrils affecting cellular integrity, which has been postulated to be associated with meat tenderisation (Huff-Lonergan et al., 1996).

Several studies have identified associations between genetic variation in *Ankyrin* genes and particular muscle phenotypes. For example, recent research has demonstrated that *Ankyrin* repeat domain 6 (*ANKRD6*) is associated with cross-sectional area in human muscle (Van Deveire et al., 2012). Expression of *Ankyrin* repeat and sterile alpha motif domain containing 1B (*ANKS1B*) has been found to be highly up-regulated in pork muscle high in fat (Hamill et al., 2012). In

Abbreviations: ANK1, *Ankyrin 1* gene; SNP, Single nucleotide polymorphism; LTL, *Longissimus thoracis et lumborum* muscle; SM, *M. semimembranosus* muscle.

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addition, the expression of *Ankyrin* repeat domain 1 (*ANKRD1*) was found to be correlated with ultimate pH in porcine muscle (Damon et al., 2013). SNPs in porcine *ANK1* demonstrated associations with water-holding capacity, pH, shear force and intramuscular fat (IMF) (Wimmers et al., 2007). Furthermore, twelve SNPs have been identified in 761 bp of the porcine *ANK1* promoter, two of which were associated with drip loss and IMF. Following haplotype construction, two haplotypes were correlated with drip loss and IMF (Aslan et al., 2012). The promoter region of bovine *ANK1* has also been associated with meat quality traits (Aslan, Sweeney, Mullen, & Hamill, 2010). Seven novel SNPs were detected in a 1.1 kb region of the *ANK1* promoter. Three SNPs within this region were correlated with meat tenderness and texture and two haplotypes were linked to increased firmness and tenderness. Correlations with increased IMF and juiciness were also identified for another haplotype in the same study (Aslan et al., 2010).

While the promoter region of bovine *ANK1* has been previously studied in relation to meat quality, no research has been carried out on the coding regions of this gene. The overall objectives of this study therefore were the following: 1) to identify novel SNPs in the coding region of the bovine *ANK1* gene and construct haplotypes, 2) to characterise these exonic SNPs and use *in silico* approaches to predict their influence on protein structure and, 3) to assess their putative association with economically important beef quality traits.

2. Methods

2.1. RNA extraction and cDNA synthesis

Isolation of total RNA was performed using Trizol® reagent (Sigma-Aldrich Corp., St. Louis, MO, USA) and TissueLyzer™ (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. In order to remove genomic DNA, the extracted RNA was treated with DNase I (Qiagen, Hilden, Germany) at room temperature for 10 min. Quantity and purity of the RNA were determined using NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Samples with a 260/280 ratio of ≥2.0 were regarded as acceptable for cDNA synthesis. 1 μg of total RNA and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) in a final volume of 20 μl, were used for a random hexamer primed cDNA synthesis, according to manufacturer's protocols.

2.2. cDNA sequencing

Six sets of overlapping primers (ANK1850A to ANK1850F) were designed using Primer3 (http://primer3.ut.ee/) (Table 1) complementary to 3 kb of the bovine *Ankyrin 1* coding region (Accession No. AF222766.1). cDNA generated from the *Longissimus* muscle was used as template (n = 32 Charolais cattle, aged 20 months at slaughter).

PCR reaction components are described in Table 2. Despite extensive modification of PCR reaction conditions (e.g. through an alteration of thermal cycling conditions, primer and $MgCl_2$ concentrations, addition of adjuvants such as DMSO) ANK1 850A PCRs could not be optimised. For the other primer sets, optimised cycling conditions were as follows: initial denaturation at 95 °C for 2 min 15 s, then 35 cycles of 95 °C for 45 s, annealing for 45 s (58 °C for 850B, 55 °C for 850C and 850D, 56 °C for 850E, 53 °C for 850F), and 72 °C for 1 min 15 s, subsequently final extension of 72 °C for 10 min.

Table 1
Forward and reverse primers for PCR amplification of Ankyrin 1 cDNA.

	Coverage (bp)	Forward primer	Reverse primer
850A	33–891	CTTCACTTGGGGAACTCGTC	CCTTTCGTGCCGTAGTTGAT
850B	771–1623	TGCTGGAAAACGGAGCTAAT	ATGAAGGAGGCTACGTGGAG
850C	1442–2197	GCCAAGGTGCTTCTGGATAA	AGGTGTGTAGCCGTTCAAGG
850D	2052–2760	AGCACGATGCACACCCTAAC	GTTTCGTCGGTGACAACCTT
850E	2622–3346	CGGACATTGTGACACTGCTT	GACAGGGCTCAGAAACTGGA
850F	3116–3733	TTCCTGGTGAGCTTCATGGT	GACCAGCTCATCGGGTACAG

PCR products were subjected to electrophoresis on 1.5% agarose gels and visualised using ethidium bromide staining in the ChemiDoc Gel Documentation System (Biorad, Hercules, CA, USA). The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN Ltd.) prior to sequencing of the purified PCR products (Eurofins, MWG-Biotech). Chromatograms were analysed using Chromas Lite software (Rothganger, Weniger, Weniger, Mellmann, & Harmsen, 2006).

2.3. In silico analysis of the cSNPs

In order to establish the location of observed SNPs on particular exons, sequences were aligned with reference *Ankyrin 1* cDNA (AF222766.1) using DNA Star-Lasergene SeqMan software (Kumar & Blaxter, 2010). The *Ankyrin 1* cDNA was then aligned with bovine chromosome 27 (chr27:38825166-38924755) (October, 2011, UCSC) to map the position of introns and exons relative to the genomic sequence.

The ExPASy translate tool (http://web.expasy.org/translate/) was used to carry out an in silico translation to determine whether and how the novel SNPs and exon would result in a change to the amino acid composition (Artimo et al., 2012).

2.4. Genotyping of tagging SNPs and meat quality phenotypes

Based on heterozygosity in the purebred Charolais cDNA samples, five coding SNPs (cSNPs) were selected for genotyping in a larger crossbred cattle population to facilitate association analysis.

DNA from commercial steers and heifers representing four EUROP grade classifications (O4L, R4L, O4H and R4H) slaughtered at an average age of 29 (±0.54) months in two commercial abattoirs was analysed. Crossbreeds including genetic contribution from Charolais, Limousin, Angus, Belgian Blue, Hereford, Holstein–Friesian were analysed and were classified as either dairy cross with major contribution from Holstein–Friesian, or beef cross. DNA from 158 crossbred cattle was analysed. As described previously in Maher, Mullen, Moloney, Buckley, and Kerry (2004), and Reardon, Mullen, Sweeney, and Hamill (2010), meat quality data was available for each of these animals for a number of traits measured on *M. longissimus thoracis et lumborum* (LTL) and *M. semimembranosus* (SM) muscles. Meat quality analysis is described in detail in Maher et al. (2004). Briefly, ultimate pH of *M. Longissimus thoracis et lumborum* (LTL) and *M. semimembranosus* (SM) muscle was measured between the 12th and 13th rib of the LTL muscle and in the ventral area of the SM muscle using a calibrated portable pH meter (Orion Research Inc., Boston, MA). Meat colour was recorded through cling film at three locations on each steak on the cut face of the LTL with a Ultrascan XE spectrophotometer (Hunter Associates Laboratory Inc., Virginia, USA) on day 2 *post mortem* after 3 h blooming, using Hunter L* (lightness), a* (redness) and b* (yellowness) colour scale (Hunter, 1972). The Ultrascan wavelengths recorded ranged from 360 to 750 nm in 10 nm intervals with diffuse illumination (D_{65}, 10°), 8° viewing angle, with 25-mm port size and specular components were excluded (Hunt, Kropf, & Morgan, 1993). Steaks aged 14 days *post mortem* were trimmed of external fat, dried with a paper towel and weighed. They were placed in plastic bags and immersed in a water bath (Model Y38, Grant Instruments Ltd, England) at 72 °C until they reached a core temperature of 70 °C. The samples were allowed to cool to room temperature and reweighed. The cook loss was expressed as a percentage of the raw weight of the steak. After cook loss was determined, the samples were used to measure Warner Bratzler shear force according to AMSA guidelines. Briefly, six cores of 1.25 cm diameter were obtained from each sample. The cores were cut in parallel to the longitudinal orientation of fibres and sheared using Instron model 5543 and data was analysed using Blue Hill software (Instron Ltd., Buckinghamshire, UK). To determine intramuscular fat content, samples frozen at −20 °C on day 2

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