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SREBP pathway genes as candidate markers in country ham production

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Abstract
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Keywords
Pig, SREBF1, MBTPS1, SCAP, SNP

Disciplines
Agriculture | Animal Sciences | Genetics and Genomics | Meat Science

Comments
This article is published as Renaville, Benedicte, Kimberly L. Glenn, Benny E. Mote, Bin Fan, Kenneth J. Stalder, and Max F. Rothschild. "SREBP pathway genes as candidate markers in country ham production." *Italian Journal of Animal Science* 9 (2010): e7. doi: 10.4081/ijas.2010.e7. Posted with permission.

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Italian Journal of Animal Science

ISSN: (Print) 1828-051X (Online) Journal homepage: http://www.tandfonline.com/loi/tjas20

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To cite this article: Benedicte Renaville, Kimberly L. Glenn, Benny E. Mote, Bin Fan, Kenneth J. Stalder & Max F. Rothschild (2010) SREBP pathway genes as candidate markers in country ham production, Italian Journal of Animal Science, 9:1, e7, DOI: 10.4081/ijas.2010.e7

To link to this article: https://doi.org/10.4081/ijas.2010.e7

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Published online: 22 Nov 2017.

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PAPER

SREBP pathway genes as candidate markers in country ham production

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Abstract

Country hams are dry-cured products from the Southeastern region of the USA. This high value product requires quality fresh meat to avoid later processing problems. The marker *SREBF1* is a transcription factor involved in the regulation of fatty acid synthesis and anti-oxidative enzyme transcription. The *SREBF1* gene and its regulators, *SCAP* and *MBTPS1*, were investigated for associations with several meat quality traits in country hams. After single nucleotide polymorphism (SNP) identification, PCR-RFLP tests were designed for one polymorphism in each of the three investigated genes. Meat quality and physical traits were collected on 299 fresh hams. Significant associations were found with ham yield (*MBTPS1*, P<0.05 and *SREBF1*x*MBTPS1*, P<0.05) and ham circumference (*MBTPS1*, P<0.05 and *SREBF1*x *MBTPS1*, P<0.01), Hunter A colour score on fresh meat (*SREBF1*, P<0.05), Hunter B colour score on cured meat (*MBTPS1*, P<0.05 and *SREBF1*x*MBTPS1*, P<0.01), moisture (*SCAP*x*MBTPS1*, P<0.05) and salt percentage (*SREBF1*x*SCAP*, P<0.05). Our findings provide initial evidence that SNPs in *SREBF1*, *SCAP* and *MBTPS1* are associated with some country ham quality traits. Breeders could use these gene tests to improve their animals, which would in turn improve country ham processing and other desired production goals.

Introduction

Country hams are American dry-cured hams produced throughout the Southeastern part of the U.S. in states like Kentucky, Tennessee, North Carolina and Virginia. These country hams differ from the traditional dry-cured hams produced in Italy and Spain in that they utilize considerably shorter curing periods and the inclusion of a smoking step after curing (Ramos *et al.,* 2007). In 2005, approximately 3.4 million country hams were processed and the retail value of these hams exceeded 340 million dollars (Stalder *et al.,* 2006). This production requires high quality meat as inconsistent pork quality can often lead to variation in water holding capacity (yield) and muscle colour of dry-cured hams (Stalder *et al.,* 2005).

Sterol regulatory element binding proteins (SREBPs) belong to the original basic helix-loop-helix leucine zipper family of transcription factors (Eberle *et al.,* 2004). Three SREBPs have been characterized: SREBP-1a, -1c, and -2, which stimulate transcription of more than 30 genes involved in the uptake and synthesis of cholesterol, fatty acids, triglycerides, and phospholipids (Horton *et al.,* 2003). The binding protein SREBP-1c preferentially activates lipogenic genes, whereas SREBP-2 acts more specifically on cholesterol biosynthesis genes (Amemiya-Kudo *et al.,* 2002). Furthermore, SREBP-1a regulates both metabolic pathways. The same gene (*SREBF1*) encodes for SREBP-1c and SREBP-1a (Shimano, 2001) but this last protein is a more potent transcriptional activator than SREBP-1c, due to its longer NH2-terminal transactivation domain (Eberle *et al.,* 2004).

The precursors of the SREBPs are retained in the ER membranes through a tight association with the SREBP cleavage activating protein (*SCAP*) (Nohturfft *et al.,* 1998). Under the appropriate conditions, *SCAP* escorts the SREBP precursors from the ER to the Golgi apparatus where two functionally distinct proteases, site 1 protease (S1P, locus symbol: *MBTPS1*) and site 2 protease (S2P), sequentially cleave the precursor protein releasing the active nuclear isoform of SREBP (nSREBPs) in the cytoplasm (Wang *et al.,* 1994; Sakai *et al.,* 1998).

The objective of this paper was to identify SNPs in the pig *SREBF1*, *SCAP* and *MBTPS1* genes and evaluate whether these SNPs are associated with country ham quality parameters.

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Key words: Pig, *SREBF1*, *MBTPS1*, *SCAP*, SNP.

Acknowledgments: the authors would like to thank Dr. Steven Lonergan and members of the Rothschild lab for their assistance. The authors appreciate the cooperation and help provided by Clifty farms.
Bénédicte Renaville's stay in Animal Science department was supported by LR 11/2003 art.11, 200502425001 and LR 26/05 art. 23 and the Ensminger Fund and State of Iowa and Hatch funding.

Received for publication: 28 January 2009.
Accepted for publication: 12 June 2009.

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Licensee PAGEPress, Italy
Italian Journal of Animal Science 2010; 9:e7
doi:10.4081/ijas.2010.e7

Materials and methods

Animals and ham processing

Hams were obtained from two pork harvesting facilities that routinely supply fresh hams to Clifty Farm Country Hams (Paris, TN, USA). All hams (321) were derived from commercially crossbred individuals, but information regarding the individual contribution of the breeds used in establishing the cross was not available. Since pigs were not tracked during the harvesting process or cutting of the carcass into primal cuts, including into hams, it was not possible to determine the sex of the animals from which the hams were derived. In order to avoid sampling the same animal twice, all hams were collected from the left side of the carcass only. In order to minimize variation due to initial ham weight, only hams between 8.5 and 10.5 kg were utilized in this study. Despite the large number of hams sampled, hams were processed for only two days, in order to minimize day of harvest effects.

Fresh ham evaluation

On each fresh ham, several physical and quality traits were recorded, including weight, circumference (measured using a flexible cloth measuring tape around the section of each ham presenting the greatest circumfer-

ence), depth (measured at the thickest part of the ham), objective colour scores, marbling and firmness scores. The latter two traits were evaluated using the U.S. National Pork Producers Council guidelines (NPPC, 2000). All these traits were evaluated on the semimembranosus muscle of each ham face at approximately 48 hours after slaughter. Objective colour scores were measured using a Minolta Chroma Meter (Ramsey, NJ, USA) with a 50 mm aperture, using Standard Illuminant C light source and 0° viewing angle geometry. The Minolta Chroma Meter was calibrated against a white tile standard prior to use. The values recorded included Minolta and Hunter L, a, and b scores and were only recorded in the first day of data collection because of machine malfunction on day 2. Since the Minolta and Hunter measures of colour often correlate closely with each other (Stalder *et al.,* 2005), only the results for Hunter scores are presented here. In addition, a sample of the *semimembranosus* was collected from each ham to obtain pH, lipid percentage and dry matter percentage. Approximately 75 g of the *semimembranosus* sample collected was homogenized using a standard food processor until it was finely ground. For pH determination, the pH meter (model IQ150, IQ Scientific Instruments, CA, USA) probe was inserted, after calibration, into the ground ham sample and two pH values were obtained from each sample and averaged. Lipid percentage was determined using a modified lipid extraction procedure based on the method described by Folch *et al.* (1957). In order to calculate dry matter, a sample of ground pork (1 g) was incubated at 80°C and weighed several times until the sample reached constant weight and was considered dry. Dry matter was then expressed as a percentage of initial sample weight.

Dry-cure processing

All hams were processed following standard commercial curing procedures in place at Clifty Farm Country Hams, including application of a salt mixture, curing time, curing temperature and humidity. The curing mixture included salt, sodium nitrate, sugar and other spices, and was applied to all hams. Afterwards, the hams were refrigerated at approximately 2.5 to 4.0°C for 5 days. Then, the hams were re-salted and refrigerated again for 44 days. Upon completion of the initial curing process, the hams were washed and then dried in chambers for a period of 20 days. These chambers were at 15°C and 58-60% relative humidity. The final step included smoking for 8 days at a temperature of 38°C. The general curing procedures adopted for country hams have been described previously (Stalder *et al.,* 2006). In order to maintain individual ham identification through the curing process, each ham was individually identified using a commercially available tagging system (Laser®3™, Koch Supplies, North Kansas City, MO, USA).

Cured ham evaluation

Upon completion of the curing process, all hams were weighed and yield was calculated as the weight of the cured ham divided by the weight of the fresh ham. Slices (9 mm thick) from each ham were cut perpendicular to the femur using a band saw. Afterwards, the same objective colour scores described above were evaluated on the *semimembranosus* muscle from a slice obtained from approximately the centre of each cured ham. The *semimembranosus* was chosen to be evaluated after the completion of the curing process, since the face of the ham is extremely dry due to curing and is not representative of the quality found in the high value centre cuts. A sample of each slice was retained for further analytical determinations including moisture and salt content. First, the cured ham samples were finely ground using the procedures described above. Moisture was determined from a 50 g ground sample using the procedures described for evaluating fresh ham dry matter percentage. The salt content was calculated using 10 g samples analyzed with a Sodium Ion Selective Electrode attached to a Model 225 pH-ISE meter (Denver Instrument Company, Arvada, CO, USA) with a standard curve verified by AOAC (1990) standard method.

Molecular genetic marker evaluation

The DNA was extracted from the fresh muscle samples using a standard DNA extraction method. In order to account for genes that are known to have a major impact on pork quality, all samples were initially genotyped for the porcine stress syndrome gene (*RYR1* – ryanodine receptor 1 gene, usually indicated as the HAL 1843™ mutation) and for the RN- or Napole genotype using the procedures described by Fujii *et al.* (1991) and Milan *et al.* (2000), respectively. New genetic markers were developed using single nucleotide polymorphisms (SNPs) identified in *SREBF1*, *SCAP* and *MBTPS1*. Initially, primer sets were used for each of these genes in order to amplify PCR fragments from each gene. *SREBF1* primer set (Table 1) was designed from the corresponding human *SREBF1* exon 6 genomic sequence, *SCAP* primer set was described in Qiu *et al.,* (2006) (Forward primer: GCAGCGCCGGGACAGTGG; reverse primer: TGATGCCCGAGGCCACCTCCTC) and amplified intron 13 of the *SCAP* gene. *MBTPS1* primers were designed from the consensus regions of exon 5 (Forward primer: TGAAGGAGAGAACCAACTGGA) and exon 6 (reverse primer: CTGCATCTGGAGCAAATCCT) of the human (accession numbers in ensemble ENSE00000945694 and ENSE00000945695) and mouse (accession numbers: ENSMUSE00000319523 and ENSMUSE00000319518) *MBTPS1* gene. After PCR optimization of these primer sets, PCR products were sequenced for each gene using an ABI automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). The genomic sequences of the amplified fragments have been deposited into GenBank, and accession numbers are FJ904278, FJ904279 and FJ904280 for *SREBF1*, *SCAP* and *MBTPS1*, respectively. The sequence comparisons between animals were used to identify SNPs. The sequences were analyzed using Sequencher software version 3.0 (Gene Codes, Ann Arbor, MI, USA). In order to ensure that the sequences obtained came from the investigated genes, they were blasted against human and mouse genomes. After identification of the polymorphisms present in each gene, new primer sets were designed in order to create PCR-RFLP tests for those polymorphisms found within recognition sites of restriction enzymes. The sequences of these primer sets for each gene are presented in Table 1. These PCR-RFLP tests were used to genotype the whole dataset. Amplifications were performed using 12.5 ng of porcine DNA, 1x PCR buffer, 2.5 mM of MgCl2, 0.125 mM each dNTP, 0.3 mM of each primer and 0.35 U Taq polymerase (Promega, Madison, WI, USA). The PCR conditions consisted of an initial step at 94°C for 3 minutes followed by 40 cycles of 94°C for 30 seconds, specific annealing temperature for each primer set (Table 1) for 30 seconds and 72°C for 30 seconds. The final extension step consisted of 3 minutes at 72°C. Digestions were performed using the restriction enzyme for each PCR-RFLP test (Table 1) following the recommendations of the manufacturer. The locations of the SNPs within the genes, as well as the fragment sizes for each allele, are also presented in Table 1.

Statistical analyses

Single locus and multiple genotype associations were analyzed using the General Linear Model of the SAS software package (SAS Institute, release 9.1, Cary, NC, USA) with a model that included ham source, day of sampling and marker genotype (*SREBF1*, *SCAP*, *MBTPS1* and the two-way interactions among these markers) as fixed effects. The model used for the analysis of cured weight was dif-

ferent because initial ham weight was included as a linear covariate. For the objective colour scores determined on the fresh hams, the model included only ham source and marker genotypes, because these traits were collected only on the first day of sampling. Information on other effects, such as sex or sire, was not available for the dataset analyzed. Samples from animals that were carriers or homozygous for the unfavourable allele at the RYR1 (RYR1 c.1843T allele) and *PRKAG3* (*PRKAG3* p.200Q allele) loci were removed from the dataset to eliminate major genes known to impact pork quality. Significant differences were declared when the marker genotype effect was a significant source of variation in the analysis of variance and the P-value for the difference between the least squares means for each marker genotype was less than 0.05.

Results

The initial dataset included 321 hams. However, 22 animals that were carriers or homozygous for the *RYR1* and/or *PRKAG3* unfavourable alleles were removed from the dataset before the association analyses.

Results from the analyses performed showed significant associations between *MBTPS1* and the interaction between *SREBF1* and *MBTPS1* (*SREBF1*x*MBTPS1*) with cured weight, yield and circumference (Table 2). The SNP c.736+78C>T (dbSNP accession no: ss120258599) within *MBTPS1* genotype was associated with significantly higher cured weight, yield and circumference values when compared with genotype TT (P<0.05) (Table 3). The *SREBF1* c.1158+118A>G (dbSNP accession no: ss120258595) polymorphism was associated with Hunter A scores measured on fresh meat (Table 2) with the AA genotype associated with significantly (P<0.05) redder meat (Table 3). Both *MBTPS1* and the interaction *SREBF1*x*MBTPS1* were associated with Hunter B scores of cured ham. The *SCAP*x*MBTPS1* and *SCAP*x*SREBF1* gene

Table 1. Genes analyzed, primers, PCR annealing temperature, restriction enzymes used, PCR-RFLP fragment sizes and allele frequencies in a study of the association between genetic markers and fresh and dry-cured ham processing characteristics.

Marker*	dbSNP accession No.	Primer	Primer sequence (5' – 3')	Fragment size (bp)	Annealing temp. (°C)	Restriction enzyme	Fragment sizes (bp) and allele frequency (%)
SREBF1 c.1158+118A>G	120258595	SREBF-F SREBF-R	ATG CCT GCC TGC CCT AAC GCC ATC TGT CCT CTT TGC TG	503	60	*Nla*III	503 (allele G: 29.7%) 373+130 (allele A: 70.3%)
SCAP c.2616C>T	120258596	SCAP-F SCAP-R	GTG TTG GAG GCT CAG GAG AG AGG AAA GCT GCC TTC ATC CT	381	62	*Bg*lII	381 (allele C: 60.8%) 246+135 (allele T: 39.2%)
MBTPS1 c.736+78C>T	120258599	MBTPS1-F MBTPS1-R	GAACCAACTGGACCAACGAG CGGACTCTCCAAGTCTGAGG	363	59	*Dde*l	345+18 (allele T: 74.9%) 228+117+18 (allele C: 25.1%)

*The genes analysed were: Sterol regulatory element binding transcription factor 1 (SREBF1), SREBF chaperone (SCAP) and membrane-bound transcription factor peptidase, site 1 (MBTPS1).

Table 2. P-values for the effects of *SREBF1, SCAP* and *MBTPS1* on fresh and cured ham traits.

Traits	*SREBF1*	*SCAP*	*MBTPS1*	*SREBF1xSCAP*	*SREBF1xMBTPS1*	*SCAPxMBTPS1*
Cured weight, kg	ns	ns	<0.05	ns	<0.05	ns
Yield, %	ns	ns	<0.05	ns	<0.05	ns
Circumference, cm	ns	ns	<0.05	<0.10	<0.01	ns
Lipid percentage, %	ns	ns	<0.10	ns	ns	ns
Fresh Hunter a	<0.05	ns	ns	ns	ns	ns
Cured Hunter L	<0.10	ns	ns	ns	ns	ns
Cured Hunter b	ns	ns	<0.05	ns	<0.01	ns
Moisture, %	ns	ns	ns	ns	ns	<0.05
Salt content, %	ns	ns	ns	<0.05	ns	ns

Green weight, Depth (cm), Marbling, Firmness, pH, Dry matter (%), Fresh Hunter L and b and Cured Hunter a were not significant at P<0.05.

Table 3. Genotypic least square means (± standard errors) for *SREBF1, SCAP* and *MBTPS1* in fresh and cured ham traits.

	SREBF1			*SCAP*			*MBTPS1*		
Traits	GG	AG	AA	CC	CT	TT	TT	TC	CC
Cured weight, kg	7.32±0.09	7.25±0.05	7.31±0.03	7.31±0.04	7.27±0.05	7.32±0.09	7.21±0.03^{a}	7.31±0.03^{b}	7.38±0.11^{ab}
Yield, %	78.71±0.94	77.95±0.50	78.59±0.36	78.50±0.41	78.09±0.49	78.66±0.94	77.46±0.07^{a}	78.51±0.36^{b}	79.28±1.20^{ab}
Circumference, cm	72.00±0.72	71.74±0.38	72.06±0.28	72.09±0.31	71.59±0.37	72.12±0.72	71.67±0.21^{a}	72.59±0.28^{b}	71.54±0.92^{ab}
Lipid percentage, %	2.46±0.34	2.37±0.18	2.38±0.13	2.46±0.15	2.37±0.18	2.39±0.35	2.11±0.10^{a}	2.50±0.13^{b}	2.61±0.44^{ab}
Fresh Hunter a	7.12±0.55^{a}	8.14±0.30^{ab}	8.63±0.21^{b}	8.09±0.29	7.85±0.30	7.94±0.51	8.08±0.18	8.01±0.37	7.80±0.60
Cured Hunter L	44.20±0.90	43.71±0.48	42.58±0.35	43.25±0.39	43.84±0.48	43.39±0.90	43.02±0.26	43.27±0.36	44.20±1.15
Cured Hunter b	5.86±0.19	5.65±0.10	5.52±0.07	5.60±0.08	5.67±0.10	5.75±0.19	5.47±0.06^{a}	5.66±0.07^{b}	5.89±0.24^{ab}
Moisture, %	63.86±0.59	64.65±0.31	64.55±0.23	64.19±0.26	64.00±0.31	64.88±0.59	64.48±0.17	64.13±0.23	64.46±0.76
Salt content, %	5.40±0.39	5.28±0.21	5.26±0.15	5.57±0.17	5.41±0.20	4.97±0.39	5.48±0.11	5.29±0.15	5.18±0.50

^{a,b}Mean values with different letters were significantly different; P<0.05.

interactions showed significant associations with moisture and salt percentage, respectively (Table 2). In addition, a suggestive association (P<0.1) with lipid content was also detected for SNP *MBTPS1* c.736+78C>T. For this marker, genotype TC had higher lipid content than animals with the TT genotype (P<0.05) (Table 3). No associations were found between the *SCAP* c.2616C>T (dbSNP accession no: ss120258596) polymorphism and the investigated ham traits, although the interaction of this marker with the other ones resulted in significant associations (Table 2).

Discussion

The SREBP-1 is a transcription factor which regulates the expression of various genes implicated in fatty acid synthesis such as (Stearoyl-CoA Desaturase and Fatty Acid Synthase) and in cholesterol uptake affecting the expression of the LDL receptor (Espenshade and Hughes, 2007) and HDL receptor SR-BI (Lopez and McLean, 1999). SREBP also controls adiponectin expression (Rahmouni and Sigmund, 2008), which regulates lipid catabolism. The release of the nuclear active form of SREBP is regulated by various proteins including *SCAP* and S1P (gene locus: *MBTPS1*) and these genes mediate the effects of SREBP. In Japanese Black cattle, a mutation in *SREBF1* was associated with meat fatty acid composition (Hoashi *et al.,* 2007). An association between the studied SNPs and lipid content was expected considering the importance of the SREBP pathway in the regulation of fatty acid synthesis. Indeed, an association between the *MBTPS1* gene and lipid content nearly reached significance. This lack of significance might be due to the limited number of samples used in this study. Moreover, it was not possible to correct the data for sex effect, which might have affected our results. Indeed, some authors reported that sex can influence fat content with castrated males being fatter than females (Candek-Potokar *et al.,* 2002; Edwards *et al.,* 2008; Latorre *et al.,* 2008;); others, however, found little or no effect (Barton-Gade, 1987; Cisneros *et al.* 1996).

MBTPS1 polymorphism and the interaction *SREBF1*x*MBTPS1* had significant effects on cured ham weight, ham yield and ham circumference. These effects may result from a secondary effect of the intramuscular fat content modification. Indeed, fat content modifies water holding capacity and, therefore, ham yield (Candek-Potokar *et al.,* 2002). This could also explain the effects of *SCAP*x*MBTPS1* on moisture and of SREBFx*SCAP* on salt content.

The SREBP is also known to induce expression of enzymes involved in the protection against oxidative stress like cysteine sulfinic acid decarboxylase, glutathione S-transferase and heme oxygenase 1 (HMOX-1) (Horton *et al.,* 2003; Kallin *et al.,* 2007). The *HMOX*-1 catalyzes the degradation of heme into biliverdin, which is subsequently transformed into bilirubin by biliverdin reductase. Further, *HMOX*-1, which is up-regulated by different types of cellular stresses, also presents potent antioxidant properties that are associated with the release of carbon monoxide, iron, and biliverdin from the heme molecule (Poss and Tonegawa, 1997; Otterbein *et al.,* 2003). The SREBP pathway genes (*SREBF1*, *SCAP* and *MBTPS1*) can affect the presence of anti-oxidative enzymes which might explain the observed association of SNPs with meat colour after slaughter.

The observed association of the *SREBF1* c.1158+118A>G SNP on Hunter A colour score may result from a difference in the muscle fibre type. Indeed, slow twitch fibres are more prone to use lipids instead of glucose as an energy source and thus require more myoglobin, resulting in meat which is more red in colour. Moreover, *SREBF1* is also involved in the regulation of aldolase C, an enzyme of the glycolitic pathway (Horton *et al.,* 2003), thus improving the use of glucose as energy supply by muscle fibres.

Most of the significant associations in the present study were obtained from the interaction of genes, suggesting that these genes could be associated in defining a few ham traits. Indeed, we observed significant associations of *SREBF1*x*MBTPS1* on quantity traits and cured colour, of *SCAP*x*MBTPS1* on moisture and of *SCAP*x*SREBF1* on salt content.

Conclusions

Our findings provide initial evidence that mutations in some SREBP pathway genes are associated with U.S country ham quality and therefore, demonstrate that these genetic markers could be used in selection programs in which pigs will be utilized in country ham production. They may also be useful for dry cured hams produced in other countries such as Italy, but further investigation of these genes on other dry cured ham production seems warranted.

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