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Genetic diversity in the stress regulatory gene *LUMAN/CREB3* of Yorkshire and Meishan pigs

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ABSTRACT

A candidate-gene high density SNP scanning approach was used to investigate the swine *LUMAN/CREB3* locus for polymorphisms in 232 Ontario Yorkshire and 29 Chinese Meishan pigs. Inter- and intra-breed differences in genetic diversity were characterized. In the Yorkshire breed, eight variations (three coding and five non-coding) were identified. Two LD blocks (550bp and ~4kb in length) featuring two and three haplotypes, respectively, were reconstructed. In the Meishan breed, six variations (two coding and four non-coding) and one LD block (~3kb in length) with three haplotypes were detected. This investigation may provide insight into variable stress-responsiveness among pigs.

KEY WORDS

genetic diversity, SNP, LUMAN, CREB3, pigs, stress response

INTRODUCTION

Stress is a serious problem for pork producers given the undesirable impact on performance parameters (e.g. feed intake, growth rate), reproduction, meat quality, and overall well-being of pigs (Martinez-Miro et al. 2016). How pigs respond to stress is highly variable and can be largely attributed to genetics (Murani et al. 2010). The Chinese Meishan breed is known to be docile and less active in response to environmental stress compared to the Yorkshire breed (Bergeron et al. 1996) that is commonly used in the North American pork industry. Higher basal cortisol levels have been associated with higher fat content (Foury et al. 2007) and decreased aggression (Murani et al. 2010), which are characteristic of the Meishan breed (White et al. 1995).

LUMAN, or CREB3 (cyclic AMP responsive element binding protein 3), is an important regulator of stress hormone signalling that regulates glucocorticoid (including cortisol) secretion and the glucocorticoid receptor (Penney et al. 2017; Penney et al. 2018). LUMAN has been shown to affect activity of the hypothalamic-pituitary-adrenal (HPA) axis in mice (Penney et al. 2017) and therefore provides a promising avenue for exploring the genetics underlying variable stress-responsiveness among animals, including pigs.

This study utilized a candidate-gene high density SNP scanning approach to characterize the genetic diversity that exists naturally in the swine *LUMAN* locus of Ontario Yorkshire and Chinese Meishan breeding herds. We postulate that naturally occurring variations in the swine *LUMAN* gene may underlie inter- and intra-breed variability in stress-responsiveness, and therefore may potentially be used as markers for stress-responsiveness to selectively breed pigs that are less susceptible to stress.

MATERIALS AND METHODS

This study included a total of 261 pigs, consisting of 232 purebred Yorkshire and 29 Meishan pigs. A preliminary genetic analysis was performed on 30 Yorkshire semen samples provided by Alliance Genetics Canada. Samples were selected from four farms across Southwestern Ontario, Canada. Blood samples were also collected from 202 Yorkshire pigs from Arkell Research Station's Swine Unit at the University of Guelph and analyzed for polymorphisms. The blood collection procedure was approved by the Animal Care Committee at the University of Guelph (AUP 3630) and pigs were cared for according to guidelines of the Canadian Council on Animal Care (2009). Genomic DNA (gDNA) obtained from 29 Meishan blood samples were provided by Yangzhou University (Yangzhou, China) and the Chinese Academy of Agricultural Sciences (Beijing, China). Meishan samples were obtained from two farms located in Eastern China. Pigs were selected from different families at each farm, to capture the genetic diversity within Meishan populations.

Yorkshire gDNA was extracted from blood samples using the EZ-10 spin column genomic DNA miniprep kit (BioBasic, Markham, Canada) and from semen samples using a slightly modified bead-based thiocyanate protocol (Wu et al. 2015). Meishan gDNA was extracted using a spin column DNA mini prep kit (Qiagen, Shanghai, China) and shipped on dry ice. gDNA concentration and quality was assessed using the NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, CA). Obtained gDNA remained at -20°C until use.

The genomic region of swine *LUMAN* (GenBank Accession No. NC_010443) was used to design primers with the NCBI primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Table 1). Exons, exon-flanking introns, ~350 bp of the 5' flanking region including the 5' untranslated region (UTR), and ~300 bp of the 3'UTR were amplified by polymerase chain reaction (PCR). Nested PCR was performed to enhance concentration and specificity of amplified

products, and to improve sequencing quality for polymorphism detection. PCR and nested PCR reactions were carried out in total volumes of 20 μ L and 25 μ L, containing ~50-200 ng of gDNA and ~50-100 ng of PCR product, respectively. Reactions contained 1x PCR buffer with Mg^{2+} , 200 μ M dNTP, 0.5 μ M primers, and 1U of ABM Taq Plus DNA polymerase. PCR reactions were carried out using a C1000 BioRad Thermal Cycler (Applied Biosystems, Foster City, California). PCR amplicons were visualized by agarose gel (1.5%) electrophoresis and purified using spin column purification prior to being sequenced.

PCR amplicons were sequenced using BigDye Terminator Sequencing Kit v3.1 (Applied Biosystems, The Netherlands) on an ABI 3730 DNA Analyzer. Amplicons were sequenced from both directions with nested primers for SNP confirmation. Resulting DNA sequences were aligned with a reference swine *LUMAN* sequence (GenBank Accession No. NC_010443) using the Basic Local Alignment Search Tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify misaligned bases. Sequence electropherograms were scrutinized for polymorphisms using 4Peaks by Griekspoor and Groothuis (<https://nucleobytes.com>).

Genetic diversity parameters, including genotypic and allelic frequencies as well as observed gene heterozygosity and homozygosity, were determined by direct counting according to the genotyping result. Haploview 4.2 software was used to check for conformance with Hardy-Weinberg equilibrium using an exact test (Barrett et al. 2005). Haplotypes and LD statistics were generated for each population using Haploview 4.2 software. LD was evaluated using the squared correlation of alleles at two loci (r^2) and LD plots were constructed.

RESULTS

Our analysis of the *LUMAN* locus revealed seven SNPs and one novel deletion in the Ontario Yorkshire breed and six SNPs in the Chinese Meishan breed (Table 2). All variants had been annotated in the European Variation Archive (<https://www.ebi.ac.uk/eva/>), with the exception of c.*97delC in Yorkshire pigs. In the Yorkshire breed, one SNP (c.-345G>T) was identified within the promoter region; three SNPs (c.80G>C, c.266C>G, and c.318C>A) were identified within coding regions (exons 1, 2, and 3, respectively); two SNPs (c.130-24T>C and c.611+213A>G) were identified within intronic regions (introns 1 and 6, respectively); and two variants (c.*80G>A and c.*97delC) were identified within the 3'-UTR. Missense SNP c.80G>C changes the second nucleotide within codon 27, resulting in a substitution between arginine and proline (p.Arg27Pro) within the activation domain of the LUMAN protein. Missense SNP c.266C>G changes the second nucleotide within codon 89, resulting in the substitution between serine and cysteine (p.Ser89Cys) located between the host cell factor-1 (HCF-1) binding motif and the basic region of swine LUMAN (Fig. 1). Located within the same region, synonymous SNP c.318C>A changes the third nucleotide within codon 106 but does not change the encoded amino acid (p.106Ser).

Three previously mentioned SNPs (c.-345G>T, c.80G>C, and c.130-24T>C) were also observed in Meishan pigs. Additionally, three SNPs were found only within the Meishan breed (Table 2). Two SNPs (c.435+142C>T and c.542+39C>G) were identified within intronic regions (introns 4 and 5, respectively), while one coding SNP (c.920A>T) was identified within exon 9. Both coding SNPs identified in the Meishan breed (c.80G>C and c.920A>T) are missense. As previously discussed, missense SNP c.80G>C results in amino acid replacement within the activation domain of swine LUMAN (p.Arg27Pro). Unique to Meishan pigs, missense SNP

c.920A>T changes the second nucleotide within codon 307 and results in the substitution between histidine and leucine (p.His307Leu) within the C-terminal region of the LUMAN protein (Fig. 1).

Allele frequencies for the three shared SNPs (c.-345G>T, c.80G>C, c.130-24T>C) differed between breeds (Table 2). The major T-C-C alleles, including the proline-encoding allele at missense SNP c.80G>C, occurred at a higher frequency (82.8%) in the sampled Meishan population compared to the 202 Yorkshire pigs (57.7%). Heterozygosity for the three shared SNPs was higher in Yorkshire pigs (40.0-51.0%) compared to Meishan pigs (27.6%). At SNP c.266C>G, the cysteine-encoding allele appeared at low frequencies (5.9% and 8.3%) within both Yorkshire populations, whereas all Meishan pigs featured the serine-encoding allele at this position. The minor allele frequency at c.*97delC was low within Yorkshire pigs, dropping from 25% in our analysis of 30 pigs, to 9.7% in the larger Yorkshire sample. The leucine-encoding allele at SNP c.920A>T was common in the Meishan population (62.1%), whereas all analyzed Yorkshire pigs featured a histidine-encoding allele. With the exception of c.920A>T, minor allele homozygosity for all Meishan SNPs only appeared in one Meishan pig (3.4%). All Yorkshire and Meishan SNPs had non-significant Hardy-Weinberg P-values (where $P < 0.05$) and were in Hardy-Weinberg equilibrium.

Two LD blocks, each comprised of three variants, were reconstructed in both Yorkshire populations (Fig. 1). Block 1 included variants c.-345G>T, c.80G>C, and c.130-24T>C, which spanned over 550 bp from the 5'UTR to intron 1. All block 1 variants were in high LD ($r^2 = 1.0$) and constituted two haplotypes, namely A1 and B1. While haplotype B1, which features the arginine-encoding allele at variant c.80G>C, was more frequent (63.3%) among the analyzed Yorkshire boars, haplotype A1 that features the proline-encoding allele at the same position was more frequent (57.7%) in the larger Yorkshire sample (Table 3). Block 2 spanned over 4 kb from

exon 3 to the 3'UTR. Variants c.318C>A and c.611+213A>G were in complete LD with each other ($r^2 = 1.0$) and in slightly lower LD with c.*80G>A ($r^2 = 0.68$ and 0.78 ; Fig. 1). Block 2 variants constituted three haplotypes, namely A2, B2, and C2. Haplotype B2 was more frequent among Yorkshire boars (61.7%), whereas haplotype A2 appeared more frequent in the larger Yorkshire sample (51.7%; Table 3). Haplotype C2 remained least frequent in both Yorkshire populations (5.9%-8.3%), with no C2 homozygous individuals detected. Block 1 and 2 variants were in high LD (r^2 values ranged from 0.60 to 1.0; Fig. 1) and only particular haplotypes co-segregated (Table 3). Variants c.266C>G and c.*97delC were in low LD with all other variants (r^2 values ranged from 0 to 0.20) and were not included in either reconstructed block (Fig. 1).

In the Meishan breed, variants c.-345G>T, c.80G>C, c.130-24T>C, and c.542+39C>G were in complete LD ($r^2 = 1.0$), while variants c.435+142C>T and c.920A>T were in low LD with all other variants (r^2 values ranged from 0.06 to 0.34; Fig. 1). One LD block spanning a region ~3.6 kb in length was reconstructed. This block was comprised of five variants (c.-345G>T, c.80G>C, c.130-24T>C, c.435+142C>T, and c.542+39C>G) that constituted three haplotypes (namely A, B, and C). The last SNP (c.920A>T) was located over 1.4 kb from the previous SNP (c.542+39C>G) and was not included in the block. All six possible genotypes composed from the three haplotypes were observed, however only two individuals were homozygous for the two less frequent haplotypes.

DISCUSSION

This study aimed to characterize the genetic diversity that exists within the *LUMAN* locus in Yorkshire and Meishan pigs. Our analysis revealed eight variants in the Yorkshire breed and six variants in the Meishan breed. In both breeds, the missense variant c.80G>C (p.Arg27Pro) was

found 10 residues from the LxxLL site (a protein sequence where 'L' is leucine and 'x' is any amino acid), which is important for LUMAN transcriptional activation function (Luciano and Wilson 2000). This variant could alter the interaction of LUMAN with the glucocorticoid receptor and consequently affect stress sensitivity (Penney et al. 2018). Located only 8 residues from the HCF-1 binding motif, the Yorkshire missense variant c.266C>G (p.Ser89Cys) could alter the co-localization of LUMAN with HCF-1 and translocation to the nucleus, where LUMAN acts as a transcription activator (Lu and Misra 2000). The Meishan missense variant c.920A>T (p.His307Leu) in the C-terminal region of LUMAN could alter hydrophobic interactions and protein folding (Betts and Russell 2003). Of the three missense SNP variants, p.Ser89Cys is most interesting. It is the only one that has an equivalent SNP in the human *LUMAN* gene and has a SIFT score of 0.01 (<https://sift.bii.a-star.edu.sg/>), indicating that it is predicted to be deleterious with high confidence. Variants identified in the 5' and 3' flanking regions (c.-345G>T, c.*80G>A, and c.*97delC) could alter *LUMAN* gene expression. However, variant c.-345G>T is not near known transcription factor recognition sites reported within the swine *LUMAN* promoter (Qi et al. 2009).

The complete linkage of three SNPs (c.-345G>T, c.80G>C, and c.130-24T>C) to form one LD block could be attributed to physical proximity, but co-segregation in both breeds suggests that they are functionally important. Complete LD was disrupted in both breeds by a variant (c.266C>G in Yorkshire and c.435+142C>T in Meishan) that was in low LD with all other variants. This suggests that an area of higher recombination could exist (Daly et al. 2001), following position c.130-24 in the swine *LUMAN* gene.

The presence of disequilibria suggests that LUMAN may be a target of selection. It is possible that alleles affecting LUMAN function and phenotype have been unknowingly selected

for or against during domestication, which has been coupled with decreased cortisol levels in pigs (Weiler et al. 1998), and/or current selective breeding practices including phenotypic selection. We can speculate that the more frequent haplotypes A2 and B2 in Yorkshire pigs and haplotype A in Meishan pigs may contain alleles that are advantageous over those featured in the other haplotypes. In the Meishan breed, haplotype A only differed from haplotype B at SNP c.435+142C>T but was over 2.5× more frequent. While the small Meishan sample size should be taken into consideration, it is possible that the ‘C’ allele in haplotype A is beneficial over the ‘T’ allele at this position. Larger sample sizes will provide a clearer indication of allele and haplotype frequencies within different breeding herds and could enable detection of selection-induced deviation from Hardy-Weinberg equilibrium. Further molecular studies on the gene function of *LUMAN* will help clarify the nature of the disequilibria, as other factors besides selection could also be at play.

Our characterization of the genetic diversity within the swine *LUMAN* locus of different breeds will be useful for association studies on stress responsiveness (Larson et al. 2020, submitted for publication) and could provide insight into inter-individual stress-responsiveness as well as the mechanisms by which *LUMAN* regulates stress response in pigs.

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Table 1. Primers and conditions for amplification and sequencing of swine *LUMAN* gene regions

Primer name	Sequence (5'-3')	Amplicon (bp)	PCR parameters / T _m ^a
Application: ~350 bp of 5' flanking region, 5'UTR, exon 1, intron 1, exon 2			
Forward outer	CGCTAACCCGCTCGTTCT	1251	34 cycles, 95°C 30s, 60.4°C 30s, 72°C 90s
Reverse outer	ACCCGGTCAATGTGGAATA		
Forward nested	TTCGACACTCTCGGCCACTTC	1062	35 cycles, 95°C 30s, 61.2°C 30s, 72°C 75s
Reverse nested	AGCCAGGTCCATAGTCCCCT		
Forward seq	GTAGTCCTTCCTTTTCCTAGCCC	841	64°C
Reverse seq	CTCCCCCAAACCTTCACCTCTA	1039	64.1°C
Application: intron 2, exon 3, intron 3, exon 4, ~310 bp of intron 4 (flanking exon 4)			
Forward outer	GCGTCACTTGTGTGGTGTG	1369	35 cycles, 95°C 30s, 58.3°C 30s, 72°C 90s
Reverse outer	TCCAGCTTCTTCAATGCTAGTG		
Forward nested	GAGCGATTGGGACGTAGAGG	803	34 cycles, 95°C 30s, 59.2°C 30s, 72°C 60s
Reverse nested	CCCTCCTCACTTGATGTAATCCT		
Forward seq	AGCCACTCTGACCCCTATCT	736	61.7°C
Reverse seq	CTGCCTCAGTCCTTTCTCAA	598	65.7°C
Application: ~115 bp of intron 4 (flanking exon 5), exon 5, intron 5, exon 6, ~250 bp of intron 6 (flanking exon 6)			
Forward outer	TCTCGGGGGCATTACATACAG	761	36 cycles, 95°C 30s, 61°C 30s, 72°C 75s
Reverse outer	GGTTAGGGTTCCAGCCACAC		
Forward nested	AAGCTTGAACCTCCGTTGCCT	635	34 cycles, 95°C 30s, 60°C 30s, 72°C 60s
Reverse nested	GCCACTCCAGAATCCCCATC		
Forward seq	TGATGTTACCCCTTTTCCCCAA	589	65°C
Reverse seq	TCTGCCACGTGAGGTCTTTTT	600	65.9°C
Application: ~10 bp of intron 6 (flanking exon 7), exon 7, intron 7, exon 8, intron 8, exon 9, ~300 bp of 3'UTR			
Forward outer	AGGAGGTGATCACTTGCAAAAA	1304	36 cycles, 95°C 30s, 60.6°C 30s, 72°C 90s
Reverse outer	GGACAAAGCTCCGGCCTAAA		
Forward nested	GGTGGCCTTAACAGGAGGAT	1050	34 cycles, 95°C 30s, 58.3°C 30s, 72°C 75s
Reverse nested	CAGTAAAGAGGGGTTGAGGG		
Forward seq ^b	TTGTTACATGTCAGGGTGCC	232	63.5°C
Forward seq 2 ^b	GTCTTGCTCTCTCCTTCTGTCT	781	64.2°C
Reverse seq	CCCCAGAAAGCCAGTAAGTGT	643	64.2°C

^a Only melting temperature is indicated for sequencing primers.

^b Multiple sequencing primers used for exons 7-9 due to polymerase slippage from mononucleotide T run in intron 7.

Table 2. Genetic diversity parameters of *LUMAN* variations

Variant	Reference SNP ID	Genotype frequency	Allele frequency	Obs HOM ^a	Obs HET	HWpval
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30 Yorkshire boars									
c.-345G>T	rs320607298	GG	GT	TT	G	T^b			
		0.433	0.400	0.167	0.633	0.367	0.600	0.400	0.638
c.80G>C	rs196952404	GG	CG	CC	G	C			
		0.433	0.400	0.167	0.633	0.367	0.600	0.400	0.638
c.130-24T>C	rs196959924	TT	CT	CC	T	C			
		0.433	0.400	0.167	0.633	0.367	0.600	0.400	0.638
c.266C>G	rs335228081	CC	CG	GG	C	G			
		0.833	0.167	0.0	0.917	0.083	0.833	0.167	1.000
c.318C>A	rs340240420	CC	AC	AA	C	A			
		0.400	0.433	0.167	0.617	0.383	0.567	0.433	0.863
c.611+213A>G	rs45434665	AA	AG	GG	A	G			
		0.400	0.433	0.167	0.617	0.383	0.567	0.433	0.863
c.*80G>A	rs328782843	GG	AG	AA	G	A			
		0.467	0.467	0.066	0.700	0.300	0.533	0.467	0.956
c.*97delC	no	CC	CΔ	ΔΔ	C	Δ			
		0.567	0.367	0.066	0.750	0.250	0.633	0.367	1.000
202 Yorkshire pigs									
c.-345G>T	rs320607298	GG	GT	TT	G^b	T			
		0.168	0.510	0.322	0.423	0.577	0.490	0.510	0.652
c.80G>C	rs196952404	GG	CG	CC	G	C			
		0.168	0.510	0.322	0.423	0.577	0.490	0.510	0.652
c.130-24T>C	rs196959924	TT	CT	CC	T	C			
		0.168	0.510	0.322	0.423	0.577	0.490	0.510	0.652
c.266C>G	rs335228081	CC	CG	GG	C	G			
		0.881	0.119	0.0	0.941	0.059	0.881	0.119	0.968
c.318C>A	rs340240420	CC	AC	AA	C	A			
		0.168	0.510	0.322	0.423	0.577	0.490	0.510	0.652
c.611+213A>G	rs45434665	AA	AG	GG	A	G			
		0.168	0.510	0.322	0.423	0.577	0.490	0.510	0.652
c.*80G>A	rs328782843	GG	AG	AA	G	A			
		0.228	0.510	0.262	0.483	0.517	0.490	0.510	0.902
c.*97delC	no	CC	CΔ	ΔΔ	C	Δ			
		0.817	0.173	0.010	0.903	0.097	0.827	0.173	1.000
29 Meishan pigs									
c.-345G>T	rs320607298	GG	GT	TT	G^b	T			
		0.034	0.276	0.690	0.172	0.828	0.724	0.276	1.000
c.80G>C	rs196952404	GG	CG	CC	G	C			
		0.034	0.276	0.690	0.172	0.828	0.724	0.276	1.000
c.130-24T>C	rs196959924	TT	CT	CC	T	C			
		0.034	0.276	0.690	0.172	0.828	0.724	0.276	1.000
c.435+142C>T	rs322733530	CC	CT	TT	C	T			
		0.586	0.380	0.034	0.776	0.224	0.620	0.380	1.000
c.542+39C>G	rs339549608	CC	CG	GG	C	G			
		0.690	0.276	0.034	0.828	0.172	0.724	0.276	1.000
c.920A>T	rs333971360	AA	AT	TT	A	T			
		0.207	0.345	0.448	0.379	0.621	0.655	0.345	0.249

^a HWpval: *p* value for Hardy-Weinberg-Equilibrium; ObsHET: observed heterozygosity; ObsHOM: observed homozygosity.

^b Bolded allele frequencies indicate the minor allele frequency for each variant.

Table 3. LUMAN haplotype and genotype frequencies

Block 1		Block 2		
30 Yorkshire boars				
Hap A1: TCC	0.367		Hap A2: AGA	0.300
Hap B1: GGT	0.633		Hap B2: CAG	0.617
			Hap C2: AGG	0.083
A1A1	0.167 (n=5)	A2A2	0.070 (n=2)	
A1B1	0.400 (n=12)	A2B2	0.360 (n=11)	
B1B1	0.433 (n=13)	A2C2	0.100 (n=3)	
		B2B2	0.400 (n=12)	
		B2C2	0.070 (n=2)	
		C2C2	0.0 (n=0)	
202 Yorkshire pigs				
Hap A1: TCC	0.577		Hap A2: AGA	0.517
Hap B1: GGT	0.423		Hap B2: CAG	0.423
			Hap C2: AGG	0.059
A1A1	0.322 (n=65)	A2A2	0.262 (n=53)	
A1B1	0.510 (n=103)	A2B2	0.450 (n=91)	
B1B1	0.168 (n=34)	A2C2	0.059 (n=12)	
		B2B2	0.168 (n=34)	
		B2C2	0.059 (n=12)	
		C2C2	0.0 (n=0)	
29 Meishan pigs^b				
Hap A: TCCCC	0.603	AA	0.379 (n=11)	
		AB	0.276 (n=8)	
Hap B: TCCTC	0.224	AC	0.172 (n=5)	
		BB	0.035 (n=1)	
Hap C: GGTCG	0.172	BC	0.103 (n=3)	
		CC	0.035 (n=1)	

^a Connecting lines illustrate observed co-segregations between haplotypes from different blocks.

^b Only one LD block is present in Meishan pigs.