Session 2

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SNP detection on *LHB* gene and association analysis with litter size in pigs.

G. Muñoz, A. Fernández, C. Barragán, L. Silió, C. Óvilo and C. Rodríguez.*

Dpto Mejora Genética Animal, INIA, Madrid, Spain.

**corresponding author: E-mail: gloriamm@inia.es*

Summary

The luteinizing hormone beta (LHB) plays an essential role in gametogenesis and sexual development of mammals. In the present work the *LHB* DNA was sequenced from blood samples of Iberian and Chinese-European pigs; and six single nucleotide polymorphisms (SNP) were found in Chinese-European line, located on intron 1 (SNP1, 2, 3 and 4), exon 2 (SNP5) and intron 2 (SNP6). The SNP4 was genotyped using a PCR-RFLP (*Tsp45*I) protocol and its potential effect on litter size was evaluated in the Chine-European pig population. However, no statistically significant association between the *LHB* polymorphism and litter size was found.

Introduction

The *LHB* (luteinizing hormone beta polypeptide) gene is a member of the glycoprotein hormone beta chain family and encodes the beta subunit of luteinizing hormone (LH). LH is expressed in the pituitary gland and promotes spermatogenesis, ovulation and luteinization of the ovarian follicle by stimulating the testes and ovaries to synthesize steroids (Stouffer et al., 2003). In human abnormalities in the function of LH may affect the processes of spermatogenesis and ovulation and thus result in infertility and menstrual disorders (Ramanujam et al., 2000). Roy et al. (1996) described a 1502G-A transition in the human *LHB* gene, causing replacement of glycine by serine at amino acid 102 in this protein. Subsequently, Liao et al. (1998) and Ramanujam et al. (1999, 2000) identified associations between this variation and both male and female infertility. In mammals this gene can be considered candidate for traits related to fertility and prolificacy **(**Mellink et al., 1995). The main objective of the current study was to identify polymorphisms on the *LHB* gene that might influence litter size in Iberian and Chine-European pigs.

Material and Methods

Genomic DNA samples from 5 Iberian and 4 Chinese-European pigs were isolated from blood according to a standard protocol (Sambrook et al., 1989). The sequence of the *LHB* gene from these pigs was analysed. Three overlapping fragments (F1, F2, F3) were PCR amplified with primers designed from the pig *LHB* gene sequence (GenBank accession no. D00579). Primer sequences, annealing temperatures and amplicon sizes are given in Table 1.

Table 1: Primer sequences, annealing temperatures and amplicon sizes.

FRAGMENT	AMPLIFIED REGION	PRIMERS (5´- 3´)	ANNEALING T^a(ºC)	SIZE (bp)
F1	5´ UCR-INTRON 1	GTGCCCCCATCGCGATCTCTTTAG TCCTCCGCCTGACATCTGCCATTC	65	500
F2	INTRON 1-INTRON 2	TGGGCATCTGAGGTGCTGGGGTAT TCCCGGTGCCACCTCCTGTCTTC	65	448
F3	EXON 2-3´ UCR	CAGCATCTGTGCCGGCTACTGTCC ACGCCCCAGCCCCCAGAATAGAC	65	665

Polymerase chain reactions (PCR) were performed in a 15 μl final volume, containing 50 ng of DNA, standard PCR buffer (75 mM Tris-HCl pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer and 0.4 U *Tth* DNA polymerase (Biotools, Madrid, Spain). Amplification conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 sec, annealing temperature for 30 sec and 72 °C for 45 sec, with a final extension step of 10 min at 72 °C. PCR reactions were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA). Fragments obtained were purified and sequenced in both directions in an ABI 3730 automatic sequencer (Applied Biosystems, Warrington, UK).

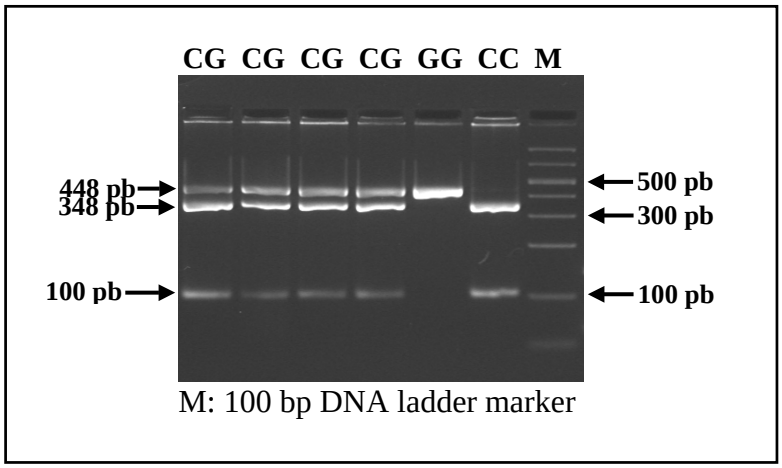
The alignment of the *LHB* sequences allowed the identification of six single nucleotide polymorphism (SNP1, SNP2, SNP3, SNP4, SNP5 and SNP6) in the Chinese-European line. Out of them, the SNP4 (C/G) at position 1549 creates a polymorphic *Tsp45*I restriction site (characteristics of restriction enzyme are shown in Table 2), that was subsequently genotyped by PCR-RFLP in Chinese-European sows for which litter size data have been recorded. Amplification products (4 μl) were digested with 2 U *Tsp45*I and BSA (200 μg/ml) in a final 10 μl volume, and genotypes were determined by

electrophoresis in 3% agarosa gels. The restriction patterns for the *G* and *C* alleles were 448 bp and 348/100 bp, respectively (Figure 1).

Table 2: Characteristics of the restriction enzyme *Tsp45*I:

Restriction enzyme	*Tsp45*I
Restriction enzyme site (5´…3´)	…G T S A C…
Buffer	NEB 1 (New England Biolabs)
BSA	200 μg/ml
Digestion T (ºC)	65

Figure 1: *Tsp45*I restriction patterns:



M: 100 bp DNA ladder marker

An statistical association analysis was performed for this SNP fitting an animal model with repeatability, using records of total number of piglets born (TNB) and number of piglets born alive (NBA) from 1,230 litters of 357 Chinese-European sows. This model included genetic line (Chinese-European), parity (1, 2, 3, 4, 5), *LHB Tsp45*I genotype (CC, CG, GG), and year-season (9 levels) as fixed effects; and the individual additive genetic effect and the permanent environmental effect as random effects. The additive effect of the SNP was measured as half of the difference between the homozygous genotypes: ([CC-GG]/2), whereas the dominance effect was estimated as the deviation of the heterozygous genotype from the mean of the homozygous genotypes (CG - 0.5 * [CC+GG]).

Results

We performed the sequence analysis of 1383 bp of this gene (positions 1030 to 2427, Genbank accession nº: D00579), including the three exons. The comparison of the *LHB* sequences from the five Iberian and four Chinese-European pigs revealed that the five Iberian pigs showed identical sequences and allowed the detection of six SNPs, four of them located on intron 1 (SNP1, SNP2, SNP3 and SNP4), one on exon 2 (SNP5) and the last one on intron 2 (SNP6) (shown in Table 3).

Table 3: Results of the *LHB* gene sequencing.

SNP-location GenBank: D00579	IBERIAN	CHINESE-EUROPEAN			
	***n=5**	**1**	**2**	**3**	**4**
SNP1 (G/A)- Intron 1- 1366	GG	AA	AA	AA	AA
SNP2 (C/G)- Intron 1- 1411	CC	CC	CG	CG	CG
SNP3 (G/T)- Intron 1- 1420	GG	GG	GT	GT	GT
SNP4 (C/G)- Intron 1- 1549	CC	CG	CG	GG	GG
SNP5 (C/T)- Exon 2- 1757	CC	TT	TT	TT	TT
SNP6 (A/C)- Intron 2- 1823	AA	CC	CC	CC	CC

*The five Iberian pigs showed identical sequences

Three out of the six SNPs were polymorphic in the Chinese-European line (SNP2, SNP3 and SNP4) and one of them, the SNP4 at position 1549 of the intron 1, was genotyped on 292 sows from this population. Allelic frequencies were 0.6 and 0.4 for *G* and *C* alleles, respectively. The values of the additive effects on prolificacy were 0.02±0.25 and 0.01±0.21, respectively, for TNB and TNA and dominant effects were 0.30±0.33 and 0.02±0.28, for TNB and TNA. No statistically significant association between the *LHB* polymorphism and litter size was found.

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