Association of Polymorphisms in Epidermal Growth Factor, Prostaglandin-endoperoxide Synthase 2 and Prolactin Receptor Genes with Semen Quality in Duroc Boars

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ABSTRACT : The quality characteristics of semen are important indicators of the fertility of a boar. Development of genetic markers for the semen quality in boars will be beneficial to the improvement of porcine fertility. We investigated the relationship between the polymorphisms of epidermal growth factor (*EGF*), prostaglandin-endoperoxide synthase 2 (*PTGS2*) and prolactin receptor (*PRLR*) genes, and semen quality traits in boars. The genomic DNA of 233 boars (157 Duroc and 86 Landrace) from a central testing station was subjected to genotyping for surveying gene frequency. The *EGF*, *PTGS2* and *PRLR* genotypes were determined using the restriction fragment length polymorphism method. Thirty-seven normal, mature Duroc boars from an AI center were also genotyped and their semen quality traits were collected. The effect of genotype on semen quality traits was analyzed by the least-squares means method using data corrected for season. The frequencies of the AA genotype of *EGF*, *PTGS2* and *PRLR* in Duroc boars were 0.14, 0.01 and 0.66, respectively. In Landrace, the frequencies of the AA genotype were 0.03, 0.09 and 0.62, respectively. Boars with the BB genotype in *EGF*, with the AB genotype in *PTGS2* and with the AA genotype in *PRLR* had significantly better semen quality with a higher percentage of normal sperm and a lower percentage of immature sperm than those with other genotypes. These findings imply that polymorphisms of *EGF*, *PTGS2* and *PRLR* genes might be used as markers for improving the semen quality of boars. ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 6 : 793-798)***

Key Words : Boar, Epidermal Growth Factor Gene, Prolactin Receptor Gene, Prostaglandin-endoperoxide Synthase 2 Gene, Restriction Fragment Length Polymorphism, Semen Quality

INTRODUCTION

Reproductive traits are some of the most economically important traits in pig production. Boars play essential roles in the genetic improvement of pigs. Malmgren and Larsson (1984) proposed that semen evaluation could be used as an indicator of the fertility of boars. The percentage of normal sperm, sperm motility and sperm concentration have frequently been used as criteria for evaluating the quality of semen (Colenbrander et al., 1993). Popwell and Flowers (2004) also showed that characterizing relationships between the quality of semen and estimates of fertility is useful in determining the differences among the fertilities of ejaculates from individual boars. The developments in genomic research have identified thousands of molecular markers. These markers include RFLP, PCR-RFLP, RAPD, AFLP, SSCP, microsatellite and SNPs (Vignal et al., 2002). Developments in the physical and genetic maps of farm animals have led to genetic improvement based on selection of marker genes. Advances in genomic research have changed and improved the production of livestock (Rothschild, 2004). Currently, the preferred way to exploit the biological information generated by genome analysis is to employ marker-assisted selection (MAS) (Georges, 2001). They are now being used or are being evaluated for use in accelerating the improvement of economically important traits in livestock, through MAS in breeding projects (Georges, 2001; Evans et al., 2003; Rothschild, 2004).

Hormone and hormone receptors are presumed to be good candidate genes for affecting reproductive traits because they are involved in the limiting steps of various reproductive pathways (Vincent et al., 1998). The candidate gene approach to improve litter size has been well documented (Rothschild et al., 1996; Vincent et al., 1998; Yun et al., 2001; Li et al., 2002; van Rens and van der Lende, 2002; Li et al., 2004; Zhang et al., 2004). The role of these factors in male reproductive functions remains largely unknown. Epidermal growth factor (*EGF*) is involved in regulating cell growth and cell differentiation (Boonstra et al., 1995). Prostaglandin-endoperoxide synthase 2 (*PTGS2*), also known as cyclooxygenase 2, is the rate-limiting enzyme in the formation of prostaglandins (Lim et al., 1997). The prolactin receptor (*PRLR*) locus has been reported to be associated with litter size of pigs (Vincent et al., 1998). The *EGF*, *PTGS2* and *PRLR* genes have been reported to be involved in the physiology of reproduction (Hadley, 1996; Lim et al., 1997) and associated with litter size (Vincent et al., 1998; Drogemuller et al., 2001). These genes have been considered candidate genes in affecting

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Table 1. Primer sequence, polymerase chain reaction products, restriction enzyme used and allele size of the genes studied

Genes[1]	Primer sequences	PCR product size (bp)	Restriction enzyme	Allele size, bp	
				A allele	B allele
EGF	Forward: 5' GAA ACA ATT CCC GTG TTC TCT 3'	1,410 or	No enzyme used	1,410	532
	Reverse: 5' TCA CTT CCA CAC CTG TAA CAT CT 3'	532			
PTGS2	Forward: 5' GTG CAC TAC ATA CTT ACC CAC TTC 3'	1,550	*Mse I*	360[2]	240 [2]
	Reverse: 5' AGG CTT CCC AGC TTT T(A/G)TA 3'				120
PRLR	Forward: 5' CGT GGC TCC GTT TGA AGA ACC 3'	163	*Alu I*	85	104
	Reverse: 5' CTG AAA GGA GTG CAT AAA GCC 3'			19	59
				59	

[1] *EGF*: epidermal growth factor; *PTGS2:* prostaglandin-endoperoxide synthase 2; *PRLR*: prolactin receptor genes.
[2] Monomorphic bands with sizes of 195, 170, 155, 150, 115, 105, 100 and 90 bp were also present.

female reproduction (Drogemuller et al., 2001; Linville et al., 2001; van Rens and van der Lende, 2002). However, few studies have addressed the involvement of these genes in male reproductive function. The purpose of this study was to investigate the relationship between the polymorphisms of *EGF*, *PTGS2* and *PRLR* genes, and the quality traits of semen in boars. The results imply that genotypes of *EGF*, *PTGS2* and *PRLR* significantly affect the semen quality of Duroc boars. These polymorphisms may be used as markers to improve semen quality traits.

MATERIALS AND METHODS

Source of animals and data collection

The samples for this study were from 2 sources: One for gene frequency survey and the other for association analysis. For the representation of gene frequency survey for the pig population in Taiwan, a total of 233 boars (157 Duroc and 86 Landrace) from a central testing station (Miaoli, Taiwan) were used. These boars were sold at the end of testing, so it is not possible for collecting semen quality traits. A total of 233 boars (157 Duroc and 86 Landrace) from a central testing station were used to survey gene frequency. Utmost effort was made to ensure that the surveyed boars were not genetically related, that is to avoid the boars with obvious genetic relationship (full-sibs or half-sibs), such that the sample was random and represented the pig population in Taiwan. For analyzing the association between gene polymorphisms and semen quality traits, we further use the boars from an AI center (Miaoli, Taiwan).

The semen quality traits were routinely recorded in the daily practice. Since the numbers of Landrace boars were limited, 37 normal, mature Duroc boars were used in the analysis. The age of the boars at the start of this study was 313.9±13.0 days. The collection frequency of semen for all boars was standardized to a frequency of twice a week. The semen quality traits were recorded during a two-year period. The semen quality measurements of boars were ranged from 2 to 24 per season. A phase contrast microscope was employed to assess the semen quality traits, including motility of sperm (MOT), percentage of normal sperm (NOR), percentage of sperm with proximal plasma droplets (PPD), percentage of abnormal sperm (ABN), concentration of sperm (CONC), volume of semen per ejaculate (VOL) and total number of sperm per ejaculate (TOT) (Huang et al., 2000). Briefly, fresh semen was dropped onto a pre-warmed (37°C) slide, on which a coverslip was overlaid. The motility was examined at 100-200X magnification, as described by Niwa (1961). The concentration of sperm was calculated using the haemacytometer method developed by Herrick and Self (1962). The NOR, PDD and ABN were calculated by examining more than 100 sperms per sample at 600-800X magnification. The abnormal sperm were defined as those with a damaged acrosome and/or morphological malformation (such as broken or curved tail). The semen quality was assessed by a single well-trained technician, to reduce variation.

Preparation and manipulation of genomic DNA

The genomic DNA of the centrally tested boars was isolated from blood using a DNA Isolation Kit for mammalian blood (Roche Diagnostics GmbH, Mannheim, Germany), as per the instructions of the manufacturer. Genomic DNA from the boars at the AI center was isolated from the sperm using a Genomic DNA Purification Kit (Fermentas, Vilnius, Lithuania), according to the manufacturer's instructions. The isolated genomic DNA was stored at -20°C until it was used in the polymerase chain reaction (PCR) for amplification of the target genes. The primers and PCR conditions of *EGF* (Mendez et al., 1999), *PTGS2* (Gladney et al., 1999) and *PRLR* (Drogemuller et al., 2001) were designed according to the methods reported elsewhere. Table 1 lists the primer pair sequences. PCR was performed using 50 ng of genomic DNA, 1×PCR buffer, 200 μM of each dNTP, 0.2 μM of each primer and 1.5 units EX Taq (for *EGF* and *PTGS2*; TaKaRa Bio Inc, Shiga, Japan) or Pro Taq Plus (for *PRLR*; Protech Technology/ Enterprise Co, Ltd, Taipei, Taiwan) polymerase in a 25 μL reaction volume. The PCR thermal cycling condition of the genes were: *EGF*: 40 cycles of 30 sec at 94°C, 30 sec at 52°C, and 90 sec at 72°C; *PTGS2*: 33 cycles of 30 sec at 94°C; 30 sec at 52°C, and 90 sec at 72°C; *PRLR*: 40 cycles of 30 sec at 94°C, 30 sec at 54°C, and 15 sec at 72°C.

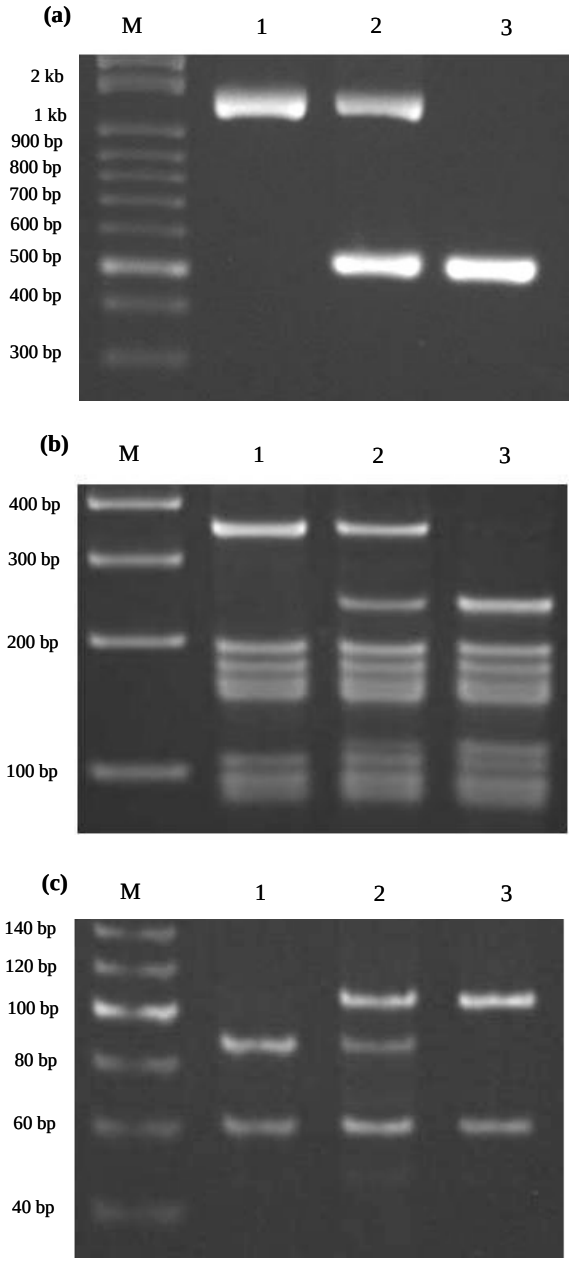

Figure 1. Restriction fragment length polymorphisms of EGF (a), PTGS2 (b) and PRLR (c). *EGF*: PCR amplification generated one or two major bands-1,410 bp (A allele) and 532 bp (B allele). *PTGS2*: Digestion of the PCR product with *Mse*I showed that a 360-bp band was present in allele A, and that bands with sizes of 240 and 120 bp were present in allele B. The monomorphic bands with sizes of 195, 170, 155, 150, 115, 105, 100 and 90 bp were present in all genotype. *PRLR*: The alleles for *PRLR* were distinguished by the presence of 85, 59 and 19 bp (A allele) and 104 and 59 bp (B allele) fragments following digestion with *Alu*I. M: DNA ladder; Lane 1: AA genotype; Lane 2: AB genotype; Lane 3: BB genotype.

Determination of genotype

The polymorphism of *EGF* can be directly distinguished by the presence of PCR product, that is the presence of 1,410 bp PCR product represent A allele, the presence of 532 bp PCR product represent B allele), and if both PCR product present the boar is heterozygote (Figure 1a). The polymorphisms of *PTGS2* and *PRLR* can not be determined directly by the PCR product, they were determined by further digestion with suitable restriction enzyme. The polymorphism of *PTGS2* was detected by digesting the PCR product using *Mse*I restriction enzyme. The A allele contained a 360-bp band and the B allele included bands of sizes 240 and 120 bp (Figure 1b). The monomorphic band sizes were 195, 170, 155, 150, 115, 105, 100 and 90 bp. The alleles of *PRLR* were distinguished by the presence of 85, 59 and 19 bp (A allele) and 104 and 59 bp (B allele) fragments following *Alu*I digestion (Figure 1c). The identity of these polymorphic sites was verified by sequencing the PCR products using an automated DNA sequencer (ABI 377, Perkin-Elmer, Foster City, CA, USA).

Statistical analysis

The genotypic frequency was calculated in both the centrally tested boars and boars in the AI center by dividing the numbers of each genotype by total boars surveyed within survey. The gene frequency of “A” allele was calculated by dividing the numbers of “A” allele (double the number of boars with AA genotype plus the number of heterozygote) to the overall alleles (double the number of boars surveyed). As each boar was evaluated at least twice during each season, the mean of the repeated measures of each boar during each season were calculated for further analysis. The normality of distribution of the semen quality traits were evaluated by UNIVARIATE procedure of SAS software package (1989). The results showed that the boars’ seasonal mean of VOL, CONC and TOTS were normally distributed, however, MOT, NOR, PPD and ABN were not normally distributed. Since the traits of MOT, NOR, PPD and ABN were presented as percentage, they were arcsine transformed. Unfortunately, the transformation did not improve the normality; the original data was thus used for analysis of variance. The effects of the season and the genotypes of each gene on the semen quality traits were analyzed by the general linear model procedure of SAS software package (1989). The statistical model included season (S), genotype (G), S×G, and boar (S×G). As the effect of season was significant for some of the semen quality traits, the significance of the differences between genotypes was determined by the least-squares means method using data corrected for season.

RESULTS AND DISCUSSION

Frequency of RFLP genotypes

One-hundred and fifty-seven Duroc and 86 Landrace boars were genotyped to evaluate the genotypic frequencies

Table 2. Frequency of genotypes of *EGF*, *PTGS2* and *PRLR* in centrally tested Taiwanese Duroc and Landrace boars

Genes[1]	Breed	No. of pigs	Frequency of pigs with indicated genotype[2]		
			AA	AB	BB
EGF	Duroc	157	0.14	0.42	0.44
	Landrace	86	0.03	0.13	0.84
PTGS2	Duroc	157	0.01	0.06	0.93
	Landrace	86	0.09	0.50	0.41
PRLR	Duroc	157	0.66	0.31	0.03
	Landrace	86	0.62	0.29	0.09

[1] As in Table 1.
[2] *EGF*: The PCR amplification generated one or two major band, 1,410 bp (A allele) and 532 bp (B allele); *PTGS2*: Digestion of the PCR product by *Mse*I revealed that a 360-bp band was present in allele A, and that allele B included bands of sizes 240 and 120 bp; *PRLR*: The alleles of *PRLR* were distinguished by the presence of 85, 59 and 19 bp (A allele) and 104 and 59 bp (B allele) fragments following digestion with *Alu*I.

Table 3. Effects of *EGF*, *PTGS2* and *PRLR* genotypes on semen quality traits[1] in Taiwan's Duroc boars[2]

Genotypes of genes[3]	No. of boars	VOL (ml)	MOT (%)	NOR (%)	PPD (%)	ABN (%)	CONC ($\times10^8$/ml)	TOT ($\times10^9$)
EGF								
AB	18	188.6±0.8^a	71.5±0.5	71.4±0.4^b	15.9±0.3^a	12.7±0.4	2.78±0.26	48.77±1.79
BB	19	176.1±1.0^b	73.5±0.6	77.2±0.5^a	8.9±0.4^b	14.0±0.5	2.74±0.35	46.16±2.17
PTGS2								
AB	4	215.0±1.8^a	71.8±1.1	76.8±0.9	11.0±0.7	12.2±1.0	2.09±0.24	44.50±3.99
BB	33	179.8±0.7^b	72.3±0.4	73.3±0.3	13.4±0.2	13.3±0.4	2.84±0.09	48.25±1.47
PRLR								
AA	23	185.4±0.8	73.2±0.5	77.0±0.4^a	9.5±0.3^b	13.5±0.4	2.72±0.11	48.15±1.77
AB	14	180.9±1.0	71.0±0.6	68.8±0.5^b	18.4±0.4^a	12.7±0.5	2.80±0.13	47.17±2.18

[1] VOL: volume of semen per ejaculate, MOT: motility of sperm, NOR: percentage of normal sperm, PPD: percentage of sperm with proximal plasma droplets, ABN: percentage of abnormal sperm, CONC: concentration of sperm and TOT: total number of sperm per ejaculate
[2] Least-squares means with different superscripts differ significantly, for genotypes of the same gene (a, b: $p<0.05$).
[3] As in Table 1.

of *EGF*, *PTGS2* and *PRLR* in Taiwan's pig population. Table 2 presents the genotypic frequencies of *EGF*, *PTGS2* and *PRLR*. The frequencies of the AA genotypes of *EGF*, *PTGS2* and *PRLR* in Duroc boars were 0.14, 0.01 and 0.66, respectively. In Landrace boars, the frequencies of the AA genotype were 0.03, 0.09 and 0.62, respectively. The gene frequencies of the A allele in *EGF* and *PTGS2* differed between Duroc and Landrace (0.35 and 0.04 vs. 0.10 and 0.34). For *PRLR*, the gene frequency of the A allele in Duroc and Landrace were similar (0.81 vs. 0.76).

In the 37 Duroc boars, the AA genotype of *EGF* and *PTGS2*, and the BB genotype of *PRLR* were not present (Table 3). The frequency of the A allele of *EGF*, *PTGS2* and *PRLR* were 0.24, 0.05 and 0.81, respectively. These frequencies were similar to those observed for the centrally tested Duroc boars.

The gene frequency estimates of the A allele in *EGF* and *PTGS2* for centrally tested Duroc and Landrace boars differed, but the gene frequency estimates in *PRLR* were similar (Table 2). Despite the observation that the AA genotype of *EGF* and *PTGS2*, and the BB genotype of *PRLR* were absent from the AI boars examined, the frequencies were similar to those observed in the central tested Duroc boars. The frequencies of the AA, AB and BB genotypes of *EGF* in the American Duroc herd were estimated to be 0.14, 0.38 and 0.48, respectively (Mendez et al., 1999). These frequencies are similar to those observed herein. However, the genotypic frequencies of *EGF* differed between American and Taiwan's Landrace, where AB is the main genotype in the American population but BB is the dominant genotype in Taiwanese population. The frequency of the A allele of *PTGS2* in the 14 F1 parents of the PiGMaP reference families was reported to be 0.68 (Gladney et al., 1999). The frequency of the A allele of *PRLR* in German Duroc was 0.82 (Drogemuller et al., 2001). The frequencies of the A allele of *EGF*, *PTGS2* and *PRLR* in the pig lines (a Landrace/Large White composite population) selected for litter size and ovulation rate were 0.06-0.10, 0.91-0.98 and 0.19-0.36, respectively, while those in the control line were 0.10, 0.18 and 0.52 (Linville, 2001). The allelic frequencies of *EGF* and *PRLR* in the Taiwanese pig population are similar to those in the German and American populations. However, the gene frequencies of *PTGS2* differed between the Taiwanese and Western Landrace populations. The differences between allelic frequencies may be caused by genetic drift or the small number of animals.

Effects of gene polymorphisms on semen quality traits

The effects of genotype on the semen quality traits in

Duroc boars from an AI center were evaluated to clarify the relationship between the polymorphisms of *EGF*, *PTGS2* and *PRLR* and these quality traits. The effect of season was significant for VOL, NOR and PPD. Only some significant interactions of season with genotype were observed. The interactions of season with EGF genotype and with PTGS2 were observed to be significant for VOL. There were also significant interactions of season with PRLR for NOR and PDD. All other interactions were not significant. Genotype significantly affected the semen quality traits analyzed (Table 3). Boars with the BB genotype in *EGF* exhibited significantly lower VOL and PDD but higher NOR than those with the AB genotype ($P<0.05$). The VOL of boars with the AB genotype in *PTGS2* was significantly higher than those of boars with the BB genotype ($P<0.05$). Boars with the AA genotype of *PRLR* have significantly better semen quality, which higher VOL ($P<0.05$) and NOR ($P<0.05$), and lower PPD ($P<0.05$), than those with the AB genotype. The MOT of boars with the AA genotype of *PRLR* tended to be higher than those with the AB genotype ($P<0.1$).

Some investigations have tried to find marker(s) for semen quality. Insulin-like growth factor has been demonstrated directly to affect bovine sperm motility (Henricks et al., 1998). Diamandis et al. (1999) reported that the concentration of prostaglandin D synthase in human seminal plasma is correlated with the semen quality. Our previous studies found that the levels of heat-shock protein 70 in boar spermatozoa may be associated with the quality traits of the semen (Huang et al., 2000) and also the single nucleotide polymorphisms in the 5'-flanking region of porcine *HSP70.2* are related to the semen quality traits in the hot season (Huang et al., 2002). Recent evidence further showed that primary fibroblasts overexpressing porcine *HSP70.2* confer cell thermotolerance (Chen et al., 2005). In the present investigation, the polymorphisms of *EGF*, *PTGS2* and *PRLR* were further examined to elucidate their association with the semen quality traits of boars. The results suggest that boars with the BB genotype in *EGF*, with the AB genotype in *PTGS2* and with the AA genotype in *PRLR* showed significantly or trends of higher VOL and NOR, and also lower PDD than those with other genotypes (Table 3). The *EGF*, *PTGS2* and *PRLR* genes have been reported to be associated with litter size (Vincent et al., 1998; Drogemuller et al., 2001) and have been considered as candidate genes that affect female reproduction (van Rens and van der Lende, 2002; Drogemuller et al., 2001; Linville et al., 2001). The results of this study suggested that the *EGF*, *PTGS2* and *PRLR* genes may associated with semen quality traits in boars. However, the exact roles of these genes in male reproduction need further elucidation. Although there were significant interactions of season with genotypes on some semen quality traits were observed, they were not further explored due to limited sample size. The interactions of season with genotypes, the interaction of the genotypes of two genes, and other candidate genes, such as insulin-like growth factor (Henricks et al., 1998), will be interesting issues for studies in the future. The percentages of normal sperm, the sperm motility, the volume of semen, and the concentration of sperm are common measures used to evaluate the semen quality (Colenbrander, 1993; Den Daas, 1992). These findings imply that polymorphisms in *EGF*, *PTGS2* and *PRLR* might be used as markers to improve the semen quality traits of boars.

In conclusion, the gene frequencies of *EGF* and *PRLR* in Taiwanese and Western Duroc populations were similar but the gene frequency of *PTGS2* in Landrace population was different. Genotype significantly influenced on the semen quality traits of Duroc boars. The results suggest that polymorphisms of *EGF*, *PTGS2* and *PRLR* genes might be candidate genetic markers for improving the semen quality in boars. However, further elucidation of the exact physiological role of these genes on male reproductive function by transgenic or knockout studies and validation of their usefulness by large scale field study as well as linkage analysis is warranted.

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