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Association Study Analysis of Cluster-of-Differentiation Antigen 9 (CD9) Gene Polymorphism (g.358A>T) for Duroc Boar Post-thawed Semen Motility and Kinematic Characteristics

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ABSTRACT

Cryopreservation of boar semen is continually researched in reproductive technologies and genetic resource banking in breed conservation. For evaluating the boar semen quality, sperm motility (MOT) is an important parameter because the movement of spermatozoa indicates active metabolism, membrane integrity and fertilizing capacity. Various researches have been trying to improve the quality of semen post-thawed in boar. Recently, polymorphism (g.358A>T) of cluster-of-differentiation antigen 9 (CD9) gene reported to be significant association with MOT. Also, CD9 gene was expressed in the male germ line stem cells is crucial for sperm-egg fusion, and was therefore selected as candidate gene for boar semen. This study was conducted to evaluate the pig SNP (g.358A>T) of CD9 gene as a positional controlling for semen parameters of post-thawed boar semen. To results, the g.358A>T SNP of the CD9 gene was significantly associated with the traits such as MOT, curve linear velocity, straight line velocity, average path velocity and amplitude of lateral head displacement. Particularly, the g.358A>T SNP significantly has the highest association with MOT and animals with AA genotype ($p<0.001$). Therefore, we suggest that the g.358A>T in the intron 6 region of the porcine CD9 may be used as a molecular marker for Duroc boar Post-thawed semen quality, although its functional effect was not defined yet.

(Key words : cluster-of-differentiation antigen 9, boar semen quality, polymorphism)

INTRODUCTION

Artificial insemination (AI) is widely practiced in the pig industry. AI is very useful tool to institute superior genes into sow herds with a minimal risk of disease when compared with natural mating (Maes *et al.*, 2008). There are two kinds of semen used AI in case of swine and they are extended fresh and post-thawed semen. Extended fresh boar semen is used for about 99% of AI in commercial swine production.

Cryopreservation of boar semen is continually researched in reproductive technologies and genetic resource banking in breed conservation. Many modifications of extenders and freezing procedures were developed (Johnson, 1998), including the pellet method of freezing, which was developed originally in Japan (Nagase and Graham, 1964). Various researches have been doing to improve Post-thawedd semen quality in boar. The first success of fertilization using post-thawed boar semen was reported by Polge *et al.* (1970). Use of boar Post-thawed semen is a useful technique for the purpose of genetic improvement, prevent the spread of disease, long distance transportation, preservation and conservation of genetic resources (Johnson *et al.*, 2000). However, sperm motility is low about 40 percent levels by cold shock, osmotic stress, and toxicity of cryo-extender during the process of semen cryopreservation (Flores *et al.*, 2008), and this causes that conception rate and litter size of post-thawed boar semen is lower compared with extended fresh semen (Johnson *et al.*, 1981).

In case of freezing of boar semen, their sperm freezability is relatively known as weaknesses compared with other livestock. Unlike other livestock's semen, boar semen is very sensitive to cold temperatures due to high content of unsaturated fatty acid in the sperm plasma membrane (Cerolini *et al.*, 2001), so

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viability and motility of boar sperm is known to decline (Mazur, 1984). Thus,. Despite recent methodological advances, cryopreservation exerts detrimental effects on spermatozoa resulting in impaired motility and mitochondrial function, and a loss of plasma membrane integrity (Fraser *et al.*, 2005; Johnson *et al.*, 2000). Many methods have been sought to improve the quality of post-thawed boar semen, including the identification of genetic differences linked to genes controlling semen freezability (Gunawan *et al.*, 2012; Kaewmala *et al.*,2012; Chen *et al.*, 2014; Zeng *et al.*, 2014; Diniz *et al.*, 2014).

Recently, polymorphism (g.358A>T) of cluster-of-differentiation antigen 9 (CD9) gene reported to be significant association with sperm motility (MOT) (Kaewmala *et al.*, 2011). CD9 is a member of the tetraspanin or transmembrane 4 (TM4) superfamily (Horejsi and Vlcek, 1991) and is expressed in many cell types including spermatogonial stem cells (Klassen *et al.*, 2001; Oka *et al.*, 2002). CD9 participates in several cellular processes such as cell migration, cell adhesion, and malignant metastasis (Boucheix *et al.*, 2001) and is reported to be a surface marker on mouse and rat male germ line stem cells (Kanatsu-Shinohara *et al.*, 2004). It transcribes a key protein during sperm-egg fusion in mammals (Le Naour *et al.*, 2000). CD9 is mapped to SSC5q25 (Yuberoa *et al.*, 2003) where QTL for testicular weight and somniferous tubular diameter were reported in pig (Ren *et al.*, 2009). This study was conducted to evaluate the pig SNP (g.358A>T) of CD9 gene as a positional controlling for semen parametersof post-thawed boar semen.

MATERIALS AND METHODS

1. Semen Collection

Boars were kept under uniform feeding and handling conditions in the National Institute of Animal Science, RDA, Korea. In this study, whole semen was collected from mature boars (Duroc, n=78) with ages ranging from 1.5 to 2 years by the gloved-hand technique from 2011 to 2015. Only ejaculates with a proportion of total motile spermatozoa >80% and a normal morphology >80% were included in the study.

Shortly after collection, the ejaculated semen was immediately filtered through a filter paper to remove gelatinous material and diluted at a ratio of 1 : 1 (v:v) with isothermal Beltsville thawing solution (BTS) at 38℃, and rapidly transferred to the laboratory.

2. Semen Cryopreservation

Before semen cryopreservation, the semen samples were stored at 17℃ for 24 h. After a period at 17℃ the extended semen was centrifuged at 800 × g for 15 min in a cooling centrifuge set at 17℃ After centrifugation, the supernatant was carefully removed using a vacuum pump. The remaining sperm pellets were slowly extended with a lactose-egg yolk (LEY) extender (11% [v/v] β-lactose, 20% [v/v] hen egg yolk) to yield a final sperm concentration of 1.5×10^9 cells/ml The extended semen was gently mixed and then further cooled to 5℃ for 1.5 h. Afterwards, the semen was slowly mixed with freezing extender consisting of 89.5 ml LEY extender, 9 ml glycerol, and 1.5 ml Orvus Es Paste (OEP, Nova Chemical Sales Inc., Scituate, MA. USA) at a ratio of 2:1 with extender, yielding a final concentration of 1×10^9 cells/ml at 5℃. The semen samples were packaged in 0.5 ml plastic straws (IMV, L'Aigle, France) by filling & sealing machine (IMV, L'Aigle, France) and then cooled to 5 for 1 h to glycerol equilibration. For freezing, the semen samples were cooled from 5℃ to −5℃ at 6℃ min, held at −5℃ for 30 sec while ice crystal formation was induced, then further cooled from −5℃ to −80℃ at 40℃ min and thereafter from −80℃ to −150℃ at 60℃ min using programmable semen freezer (SY-LAB Gerate GmbH, Austria). The semen straws were then plunged into liquid nitrogen (LN_2) for storage. The straws remained in the liquid nitrogen tank until the moment of the thawing for the analysis. After this, the straws were thawed by plunging a straw into a water bath at 38℃ for 20 sec, and the content of each straw was immediately resuspended in BTS (1:4, v/v) and incubated at 38℃ for 30 min.

3. Assessment of Sperm Motility

The MOT was objectively evaluated using a computer-assisted semen analysis system (CASA; SAIS SI-100, Medical Supply, Korea). Briefly, semen samples were suspended in BTS to a concentration of 30×10^6. For each evaluation, a 5 μl a liquot of sperm sample was placed in a pre-wormed markler counting chamber (Sefi-Medical Instruments, Israel) and five fields were analyzed at 38℃, assessing a minumum of 100 spermatozoa per sample. The kinematic parameters measured for each sperm samples included the proportion of total motile spermatozoa (TMS, %), the curvilinear velocity (VCL, μm/s), the straight-line velocity (VSL, μm/s), the average path velocity (VAP, μm/s), the percentage of linearity (LIN, %) (e.g., the ratio between VSL

and VCL) and the percentage of straightness (STR, %) (e.g., the ratio between VSL and VAP).

4. SNP Detection and Genotyping

Genomic DNA was extracted from post-thawed semen samples using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Porcine CD9 gene was amplified from 78 genomic DNA samples of Duroc pig and sequenced to detect polymorphic sites. PCR was performed in a volume of 20 μl containing 10 pmol each primer, 0.25 mM each dNTP, 2 μl 10× PCR buffer, 1.25 U DNA polymerase (Genet Bio, Chungnam, Korea), and 100 ng genomic DNA. The thermal cycling conditions included an initial denaturation for 5 min at 94℃ followed by 35 cycles of 30 s at 94℃, 30 s at 62℃ and 1 min at 72℃, with a final 10-min extension at 72℃ in a DNA Engine Tetrad® 2 Thermal Cycler (Bio-Rad, Hercules, CA, USA). To detect differences in the nucleotide sequences, direct sequencing of the PCR products was performed using a Big Dye Terminator Cycle Sequencing Ready Reaction Kit V3.0 (Life Technologies Corp., Carlsbad, CA, USA) and an ABI PRISM® 3730 Genetic Analyzer (Life Technologies Corp.). The sequences were compared to find SNPs using the SeqMan program (DNASTAR Inc., Madison, WI, USA). PCR primers used for direct sequencing were 5'-taatgggggaagtggaacaa-3' and 5'-cgccaatgatgtggaactt-3' (Kaewmala *et al.*, 2011).

5. Statistical Analysis

Association analysis was performed using SAS 9.13 (SAS Institute Inc., Cary, NC, USA). The following formula was used in a generalized linear model (GLM) analysis: $y_{ijkl} = \mu + G_i + S_j + P_l + e_{ijkl}$, where y_{ijkl} is the observed value, μ is the general mean, G_i is the fixed effect of genotype i, S_j is the fixed effect of sex j, P_l is the fixed effect of the period of slaughter 1 and e_{ijkl} is the random error. The results were presented as the least squares means for each group and standard errors (SEs) of the least squares means. Genotype, sex, breed were included as fixed effects in the statistical model. Differences were considered significant at $P<0.01$ and $P<0.05$.

RESULTS

An association study of the porcine CD9 SNP with post-thawed semen motility and kinematic characteristics were performed in 78 Duroc (Table 1). The CD9 SNP was amplified by PCR and directly sequenced to identify genetic variation in 78 Duroc boar population. As previously reported, one SNP site was found at g.358A>T in the intron 6 region of CD9 (Fig. 1). The allele and genotype frequencies for individual SNP in the Duroc boars are listed in Table 2. The estimated frequencies of genotypes AA, AT and TT were 0.22, 0.39 and 0.39, respectively. Hence, the T allele (0.58) was slightly more common than the A allele (0.42). As shown in Table 3, SNP was significantly associated with frozen semen motility and kinematic characteristics. The g.358A>T SNP was highly associated with

Table 1. Means, standard deviation (S.D.), sample size, ranges of traits in semen parameters of Duroc boars

Traits	Mean	S.D.	Min	Max
MOT (%)	31.58	16.19	3.82	89.37
VCL (μm s^{-1})	52.89	0.19	33.41	16.17
VSL (μm s^{-1})	25.98	3.38	14.17	56.72
VAP (μm s^{-1})	4.43	1.91	20.69	81.74
LIN (VSL/VCL)	26.51	4.16	37.11	77.58
ALH (μm)	2.28	0.59	1.14	4.11

SD: standard deviation, MOT: yielded sperm motility, VCL: curve linear velocity, VSL: straight line velocity, VAP: average path velocity, LIN: linearity, ALH: amplitude of lateral head displacement.

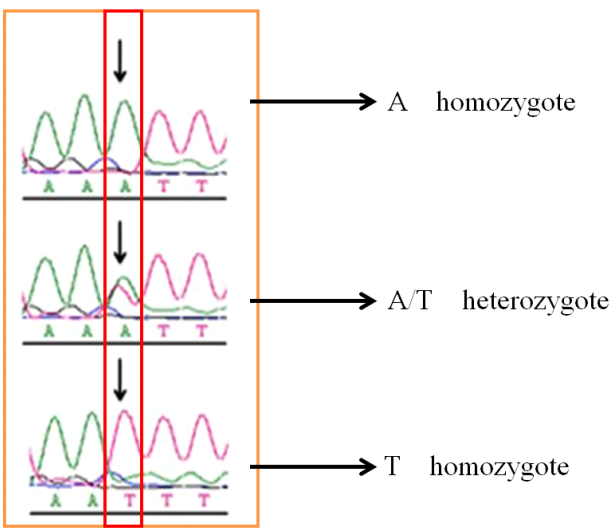

Fig. 1. Sequencing results and polymorphic sites found in the intron 6 region of CD9 gene in Duroc boars.

Table 2. Allele and genotype frequencies of *CD9* polymorphisms in Duroc boars

SNP position	Genotype frequency (*n*=78)		Allele frequency		
g.920T>C	AA (n=17, 0.22)	AT (n=31, 0.39)	TT (n=30, 0.39)	A (0.42)	T (0.58)

The number of genotyped animals and genotype frequency are shown in parentheses.

MOT (p<0.0001), VCL (p=0.005) and VAP (p=0.0037), respectively. Also, the SNP was lowly associated with VSL (p= 0.0236) and ALH (p=0.0037) respectively.

DISCUSSION

This study was conducted to evaluate the pig SNP (g.358A >T) of CD9 gene as a positional controlling for frozen semen motility and kinematic characteristics. Results for association statistical analysis in 78 animals of a commercial Duroc population were presented in Table 3. The g.358A>T SNP of the CD9 gene was significantly associated with almost traits and animals with AA genotype had high almost traits values than animals with AT or TT genotypes. Particularly, the g.358A>T SNP is the highest significantly associated with MOT and animals with AA genotype (p<0.001). CD9 gene is expressed in the male germ line stem cells is crucial for sperm-egg fusion.

In the previous report, polymorphism screening and association of CD9 with male fertility is reported in bulls by Daghigh-Kia (2007), where SNP g.95T>C in exon 9 showed a positive effect on sperm concentration and sperm motility (p<0.05) in bulls. In pig, polymorphism (g.358A>T) of cluster-of-differentiation antigen 9 (CD9) gene reported to significantly associated with sperm motility (MOT) (Kaewmala *et al.*, 2011). An association of the CD9 gene with frozen semen motility and kinematic characteristics in boars has been detected for the first time.

Animals carrying alleles A were found to have higher frozen sperm motility. This SNP was obtained in intron 6 which is subjected to less functional constraint and may change the primary structure of CD9. Though silent, it could affect CD9 function by altering the mRNA stability (Capon *et al.*, 2004). The association of such SNPs with observed traits may be explained by the influence of the intron on mRNA metabolism including initial transcription, editing and polyadenylation of the pre-mRNA, translation and decay of the mRNA product (Le Hir *et al.*, 2003). Moreover, there is increasing number of reports regarding the role of introns in regulating the expression level of a gene or tissue specific expression pattern (Pagani and Baralle, 2004).

To sum up the current results, the following conclusions can be made. In this study, the g.358A>T SNP within intron 6 of the CD9 gene was detected. For evaluating the boar semen quality, sperm motility is an important parameter because the movement of spermatozoa indicates active metabolism, membrane integrity and fertilizing capacity (Vyt *et al.*, 2004; Estienne *et al.*, 2007). From this point of view, the results of this study give us some evidence for the potential of markers used in the SNP marker-assisted selection of a Duroc boar. However, before

Table 3. Associations between SNPs of porcine CD9 and semen parameters

Gene	Traits	Genotype			*P*-value
		AA (n=17)	AT (n=31)	TT (n=30)	
CD9	MOT (%)	46.26±3.42^{a}	30.43±2.53^{b}	24.44±2.57^{b}	<.0001**
	VCL (μm s^{-1})	59.06±2.84^{a}	54.60±2.10^{a}	47.64±2.14^{b}	0.005**
	VSL (μm s^{-1})	30.40±1.81^{a}	25.23±1.34^{b}	24.26±1.36^{b}	0.0236*
	VAP (μm s^{-1})	42.16±2.32^{a}	35.72±1.72^{b}	32.02±1.75^{b}	0.0037**
	LIN (VSL/VCL)	56.75±1.98	54.05±1.46	58.08±1.49	0.1561
	ALH (μm)	2.48±0.13^{a}	2.39±0.10^{b}	2.05±0.10^{b}	0.0236*

MOT: yielded sperm motility, VCL: curve linear velocity, VSL: straight line velocity, VAP: average path velocity, LIN: linearity, ALH: amplitude of lateral head displacement.
** p<0.01, * p<0.05.

the selection of the SNP in boars, we should confirm the effects of those SNP markers by comparing the sperm motility and kinematic characteristics of boars carrying different genotypes, as the effects of an allele may vary between boar populations. In addition, since the SNP is located in intron 6, it was difficult to conclude about a direct effect of the CD9 genotypes on sperm motility and kinematic characteristics involved. Whether the association is due to the candidate gene or not require further verification. Thus, it will be of interest to continue association studies in the regions surrounding those genes.

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