B4GALT1, LHR, FSHR and *IGF2* polymorphisms and scrotal circumference in Nellore bulls

Polimorfismos dos genes B4GALT1, LHR, FSHR e IGF2 e perímetro escrotal em touros da raça Nelore

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Abstract

We assessed whether single nucleotide polymorphisms (SNPs) in the genes beta 1,4-galactosyltransferase (*B4GALT1)*, luteinizing hormone receptor *(LHR),* follicle-stimulating hormone receptor (*FSHR)* and insulin-like growth factor 2 (*IGF2*) could be molecular markers for scrotal circumference (SC) in Nellore bulls. Animals with positive (+, n = 104) and negative (-, n = 74) expected progeny difference for scrotal circumference at 365 days (EPD SC 365) were selected and their SNPs were analyzed by restriction fragment length polymorphism (RFLP). The correlation between EPD SC 365 and expected progeny difference for age at first birth (EPD AFB) was also investigated. The SNPs in *B4GALT1* and *FSHR* was not different between two groups analyzed. The CC genotype for *LHR* gene was most frequent in animals with EPD SC 365(+), whereas the TT was most frequent in the EPD SC 365(-). For *IGF2* the CT and CC were the most frequent genotypes observed in animals with positive and negative EPD SC 365, respectively. The EPD SC 365 was negatively correlated with the EPD AFB (r = 0.23). We suggest that CC and TT genotypes for *LHR* and *IGF2,* respectively, could be possible molecular markers for SC selection in Nellore bulls, that can also predict for AFB.

Keywords: Animal breeding, phenotypic selection, reproductive traits, molecular marker.

Resumo

Foram avaliados se polimorfismos de base única (SNPs) presentes nos genes beta-1,4-galactosiltransferase (B4GALT1), receptor de hormônio luteinizante (LHR), receptor de hormônio folículo estimulante (FSHR) e fator de crescimento semelhante à insulina 2 (IGF2) poderiam ser marcadores moleculares para o perímetro escrotal (PE) em touros da raça Nelore. Animais com diferença esperada de progênie positiva (+, n = 104) e negativa (-, n = 74) para PE aos 365 dias (DEP PE 365) foram selecionados e seus SNPs foram analisados utilizando a técnica de polimorfismo de comprimento de fragmentos de restrição (RFLP). A correlação entre DEP PE 365 e idade ao primeiro parto (DEP IPP) também foi investigada. Os SNPs dos genes B4GALT1 e FSHR não apresentaram diferença entre os dois grupos analisados. O genótipo CC para o gene LHR foi mais freqüente em animais com DEP PE 365 (+), enquanto o TT foi mais frequente no grupo com DEP PE 365 (-). Para o gene IGF2, os genótipos CT e CC foram mais freqüentes em animais com DEP PE 365 positiva e negativa, respectivamente. A DEP PE 365 foi negativamente correlacionada com a DEP IPP (r = -0,23). O genótipo CC para o gene LHR e genótipo TT para o gene IGF2 podem ser possíveis marcadores de PE para a seleção assistida em touros da raça Nelore, podendo ser ainda preditores para IPP.

Palavras-chave: Reprodução animal, seleção fenotípica, características reprodutivas, marcador molecular.

Introduction

Expected progeny difference (EPD) is an important tool in genetic breeding programs, since that provides information about the genetic value that a bull adds to his progeny. Furthermore, this measure can be used to compare different animals and rank them for a given trait (Henderson, 1984). Reproductive characteristic as scrotal circumference (SC) and sexual precocity have a great interest in genetic breeding.

SC is a reliable indicator of puberty in bulls (Lunstra and Cundiff, 2003) due to the advantage of being easily measured with high reliability and repeatability. It is directly correlated with important traits, such as weight (r = 0.69), probability of premature birth (PPB, r = 0.30) and age at first birth (AFB; r =-0.15) (Lirón et al., 2012; Boligon et al., 2007). There is evidence that SC is positively correlated with sexual precocity (r = 0.3), which is associated with male reproductive fertility and better sperm quality (Lirón et al., 2012).

The EPD SC at 365 days (EPD SC 365) is the most widely used phenotypic measure for the selection of the best reproducers that may indicates the sexual precocity in cattle (Olson et al., 1991; Lunstra and Cundiff, 2003). In association with phenotypic selection, molecular marker-assisted selection (MMAS), such as Single

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Nucleotide Polymorphisms (SNPs) may improve the results for reproductive traits identifying genetically superior animals in the genetic breeding programs (Marson et al., *2*008).

The beta 1,4-galactosyltransferase *(B4GALT1)*, luteinizing hormone receptor (*LHR)*, follicle-stimulating hormone receptor (*FSHR)* and insulin-like growth factor 2 (*IGF2)* are great reproductive markers that are associated with sperm quality and male fertility (de Koning et al., *2*002; Brown et al., 2009; Ikawa et al., *2*010).

Given the relationship of the genes *B4GALT1, FSHR, LHR,* and *IGF2* with reproductive traits, we evaluate the relationship of SNPs in these genes with SC in young Nellore bulls *(Bos taurus indicus)*, since the genotypic selection provide data for marker-assisted selection, facilitates phenotypic selection and increases the economic efficiency of livestock in a genetic breeding program.

Material and Methods

Samples of peripheral blood were collected from bulls *(Bos taurus indicus)* in the Nellore Brazilian Program that were divided into two groups according to there EPD SC 365. The first group comprised 104 animals with positive EPD values - EPD SC 365(+) - ranging from 0.04 to 1.28, and the second comprised 74 animals with negative EPD values - EPD SC 365(-) - between -0.72 and -0.06. DNA extraction was performed by the saline precipitation method described by Olerup and Zetterquist (1992), with modifications.

The *primers* used to obtain fragments of interest are shown in Table 1. The *FSHR*, *LHR* and *IGF2* have been previously described (Marson et al., 2008; Martins da Silva et al., 2011) and *B4GALT1* sequence was obtained from GeneBank (76179270 -76232552pb, AC_000165.1) and *primers* were designed using *GeneRunner* software. Amplification reaction mixtures consisted of 100 ng genomic DNA, 50 mM $MgCl_2$, 1× buffer (200 mM Tris, pH 8.4, 500 mM KCl), 5 picomoles of each *primer*, 0.25 mM dNTPs, and 0.2 U *Taq DNA polymerase* (Invitrogen, Life Technologies, Paisley, UK)

The temperature cycling consisted of an initial heating at 94°C for 5min; followed by 30 cycles of denaturation at 94°C for 30sec; annealing at 58°C (*B4GALT1*), 68°C (*LHR* and *FSHR*), and 60°C (*IGF2*) for 30sec; and extension at 72°C for 30sec; and a final extension step at 72°C for 10 min. The amplification products were visualized on 2% agarose gel stained with ethidium bromide (0.5 mg/ml).

Table 1. Sequences of the *primers* used to amplify the fragments of interest.

Gene/Restriction Site	Chromosome	Sequence	Reference	Product Size (Pb)
B4GALT1 RFLP/ *Nco*I	8	5'-TCCTTCACCCCTCATCACTC-3' 5'-AAACCCAGACAGCTGGTCAC-3'	-	524
FSHR RFLP/ *Alu*I	11	5'-CTGCCTCCCTCAAGGTGCCCCTC-3' 5'-CCCCCTAAGACATTTAGCCAAGAACT-3'	Marson et al. (2008)	306
LHR RFLP/*Hha*I	11	5'-CCTCCGAGCATGACTGGAATGGC-3' 5'-GCCATTCCAGTCATGCTCGGAGG-3'	Marson et al. (2008)	303
IGF2 *RFLP/MboII*	29	5'-GCCTCTCGCTGTCCTCTC-3' 5'-GAGGGGGCAGTTGAAGGAC-3'	Silva et al. (2011)	193

*bp (base of pairs), *B4GALT1* (Beta 1,4- galactosyltransferase), *FSHR* (Follicle stimulating hormone receptor), *LHR* (Luteinizing hormone receptor), *IGF2* (Insulin growth factor 2); RFLP (Restriction fragment lenght polymorphism).

Restriction Fragment Length Polymorphism (RFLP)

After amplification, approximately 100 ηg of the amplified PCR product was digested with 0.4 U of the corresponding restriction endonuclease, following the manufacturer's recommendations. The enzymes *Nco*I and *Alu*I (New England BioLabs, Ipswich, MA) were used for the analyses of *B4GALT1* and *FSHR* polymorphisms, respectively. The enzyme *Hha*I (New England BioLabs, Ipswich, MA) was used to assess the SNP in *LHR*, and

the *Mbo*II (New England BioLabs, Ipswich, MA) enzyme was used to determine *IGF2* gene polymorphism. After the amplicons were completely digested, the fragments were visualized on 10% polyacrylamide gels stained with 0.2% silver nitrate.

Statistical Analysis

The allelic and genotypic frequencies for the two groups of animals - EPD SC 365(-) and EPD SC 365(+) - were determined using the chi-square test, and the results were compared using the ANOVA (analysis of variance) test ($P < 0.05$). Pearson's correlation coefficient was used to assess the relationship between EPD SC 365 and age at first birth (AFB). Statistical analysis was performed with SAS® 9.2 software (SAS Institute Inc., North Carolina University, NC, USA).

Results

The genotypes and sequences analyzed in respect to *B4GALT1* showed only one genotype (AA) in both animals with EPD SC 365(+) and those with EPD SC 365(-). Genotypes CG and GG were identified for *FSHR*, and genotypes CC, CT, and TT for *LHR*. The genotpyes TT, CT, and CG were identified for *IGF2*.

The allelic frequency for each group - EPD SC 365(-) and EPD SC 365(+) - is presented in Table 2. The genotypic frequencies for *FSHR* polymorphism were f(GG) = 0.135 and f(CG) = 0.865 for the EPD SC 365- group. The allelic frequencies for the same group were f(C) = 0.432 and f(G) = 0.568. In the EPD SC 365(+) group, genotypic frequencies were f(GG) = 0.048 and f(CG) = 0.951, and allelic frequencies were f(C) = 0.476 and f(G) = 0.52. No significant difference was observed in genotypic frequencies between the two groups (P = 0.8454).

Table 2. Allele and genotypic frequencies for each SNP according to the phenotype of EPD SC 365.

B4GALT1/ RFLP NcoI	f (AA)	f (GA)	f (GG)	f (A)	f (G)	P value
EPD SC 365 -	1	0	0	1	0	N/A
EPD SC 365 +	1	0	0	1	0	
FSHR/ RFLP AluI	f (CC)	f (GG)	f (CG)	f (C)	f (G)	
EPD SC 365 -	0	0.135	0.865	0.432	0.568	0.8454
EPD SC 365 +	0	0.048	0.951	0.476	0.524	
LHR/ RFLP HhaI	f (CC)	f (TT)	f (CT)	f (C)	f(T)	
EPD SC 365 -	0.055	0.739	0.205	0.158	0.842	<0.0001
EPD SC 365 +	0.433	0.519	0.048	0.457	0.543	
IGF2/ RFLP MboII	f (CC)	f (TT)	f (CT)	f (C)	f(T)	
EPD SC 365 -	0.657	0.095	0.246	0.78	0.22	0.0083
EPD SC 365 +	0.371	0.238	0.39	0.567	0.433	

B4GALT1 (beta 1,4 - galactosyltransferase), *FSHR* (Follicle-stimulating hormone receptor), *LHR* (Luteinizing hormone receptor), and *IGF2* (Insulin-like growth factor 2) / RFLP (Restriction fragment lengh polymorphism); Restriction Enzymes *NcoI, AluI, HhaI, MboII*; EPD SC 365 (-) = negative, EPD SC 365 (+) = positive; f (allelic or genotypic frequencies). $P < 0.05$.

LHR was polymorphic, with CC, CT, and TT genotypes, with genotypic frequencies of 0.055, 0.739, and 0.205, respectively, in the EPD SC 365- group. Allelic frequencies were f(C) = 0158 and f(T) = 0842. In the EPD SC 365(+) group, the frequencies were f(CC) = 0.433, f(TT) = 0.519, f(CT) = 0.048, f(C) = 0.457, and f(T) = 0.543. Genotypic frequencies were significantly different between EPD SC 365- and EPD SC 365(+) ($P < 0.0001$). Thus, for the SNP located in the *LHR* gene, the CC genotype was most frequent in animals with EPD SC 365(+), whereas TT was most frequent in the EPD SC 365(-) group ($P < 0.0001$).

Regarding *IGF2* polymorphism, the EPD SC 365- group showed the following genotypic frequencies for CC, CT, and TT: 0.657, 0.095, and 0.246, respectively. The allelic frequencies for *IGF2* were f(T) = 0220 and f(C) = 0.780. In the EPD SC 365(+) group, genotypic frequencies were as follows: f(CC) = 0.371, f(TT) = 0.238, and f(CT) = 0.390; and allelic frequencies were f(C) = 0.567 and f(T) = 0.433. Both genotypic frequencies were significantly different between the analyzed populations. The genotype CT had the highest frequency in the EPD SC 365(+) group, whereas the CC genotype was the most frequent in the EPD SC 365(-) group (P = 0.0083).

As EPD is directly related to AFB (Araujo Neto *et al. 2*011), we verified the correlation between EPD SC 365 and EPD AFB for the 178 animals under investigation. We observed that these characteristics were negatively correlated (r = -0.23; P < 0.01).

Discussion

In the present study, we demonstrated that the polymorphisms evaluated in the *LHR* and *IGF2* could be used as SC indicators, since genotypic frequencies were significantly different between groups EPD SC 365(+) and EPD SC 365(-). On the other hand, the polymorphisms studied in *B4GALT1* and *FSHR* was not different between the studied groups.

Many SNPs in different genes have been used as molecular markers of reproductive traits in bovines. In Holstein bulls, studies in the *STAT5A*, *FGF2*, and *PGR* genes confirmed the association of SNPs in these genes with reproductive traits (Cochran et al., 2013). In Jersey cows, SNPs in *CWC15* were correlated with reproductive efficiency (Sonstegard et al., 2013). In addition, it has been reported that polymorphisms located in *LIN28B* and *KISS1* in humans are associated with puberty (Tommiska et al., 2011; Park et al., 2012).

The use of molecular SNP type markers complements phenotypic selection by EPDs, making collection of the data more efficient, accurate and reliable (Marson et al., 2008). *B4GALT1* is conserved in human, dogs, chickens and mice and is involved in the maturation of sperm, sperm motility, sperm binding to the zona pellucida, and the development of secondary sexual characteristics. However, we found only one genotype (AA) in our population, so this polymorphism should not be a great molecular marker of SC in Nellore bulls, as it shows no variation. Other studies using the same gene in Holstein bulls observed allelic variation, but this was related more to milk production than to sexual maturation (Shahbazkia et al., 2012).

Since the molecular markers identified in the present study can be used to select for SC, they could also drive the selection of animals with higher semen quality, resulting in higher sperm motility (60–80%) and better morphology. In addition, SC is genetically correlated to libido and AFB in bovines (Silva et al., 2011). We observed a negative correlation between EPD SC 365 and EPD AFB (r = -0.23), wherein, a high EPD SC 365 values predict that progeny of these animals will have a lower age at first birth. These results are consistent with those of a previous study in which SC 365 was negatively correlated to AFB (Boligon et al., 2007).

The TT genotype (f (TT) = 0.519) observed with high frequency in EPD SC 365(+) group, suggests that this genotype may be a potential molecular marker for SC in the population under investigation. Similarly, the CT genotype for SNPs in *IGF2*, showed a frequency of f (CT) = 0.390 in the EPD SC 365(+) group. SC has been widely used in herd genetic enhancement programs because it is a very important to reproductive traits (Lunstra and Cundiff, 2003). The high heritability (0.50) for SC indicates a considerable additive genetic variability for this trait (Cyrillo et al., *2*001). Thus, superior semen quality in livestock indicates a rapid return on investment in the maintenance of a breeding animal, especially when this semen is used for artificial insemination and there are other traits of interest present in the same animal that ensure its intensive use (Silva et al., 2011).

The SNP in the *FSHR* gene showed no difference between the studied groups (P = 0.8454), indicating that this SNP does not assist in the identification of bull precocity. Although other studies demonstrate the use of the same polymorphism as a molecular marker for economic characteristics of interest (Marson et al., 2008), this result can be attributed to sample size or breed. Further studies are therefore required to confirm these findings.

The polymorphisms of the *LHR* and *IGF2* genes under investigation are potential markers of SC due to their genotypic distribution and involvement in sperm maturation (Brown et al., 2009). These data corroborate with the choice of *IGF2* gene as a marker of sexual precocity when compared to *IGF1* gene, since the *IGF2* influences important growth traits in cattle (de Koning et al., 2002). This gene encodes a potent fetal mitogen that stimulates cell proliferation (Chao and D'Amore, 2008) and is considered to be an important stimulator of mammalian development (Brown et al., 2009). Thus, *IGF2* gene may be one of the responsible for the growth and formation of the scrotal circumference, reflecting the values of EPD SC 365.

The CC polymorphism found on *LHR* in our work may be related to greater SC, as it was predominantly present in animals with EPDSC 365(+) (P < 0.0001). This result is consistent with those of other studies that have shown positive results for the use of *LHR* gene polymorphisms as markers for reproductive traits (Marson et al., 2008; Lirón et al., 2012). Studies have shown associations of *IGF2* with phenotypes of enhanced growth and development of the animals, making the associated SNP a potential molecular marker for these characteristics (Foulstone et al., 2005). In the present study, the CT genotype was the best selector of

animals with greater SC (P = 0.0083). A similar study using the Angus breed selected the genes *GNRHR, LHR,* and *IGF1,* and associated their polymorphisms with the onset of puberty in male cattle. Specially, *IGF1* was found to be a regulator of the early onset of puberty, and was involved in the events that precede and initiate puberty in bull calves (Lirón et al., 2012).

Conclusion

SNPs located in *LHR* and *IGF2* genes may be molecular markers of higher SC, and consequently may be used to select sexually premature Nellore bulls. Additionally, the strong negative correlation between EPD SC 365 and EPD AFB confirms that the use of this EPD in genetic breeding could also result in a decrease of the intergeneration interval.

The present results, associated with other findings in genetics breeding can contribute with the phenotypic selection of the best breeding animals and suggests the use of specific SNPs as potential markers for the EPD SC 365 trait.

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