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Polymorphism of somatostatin gene and its association with growth traits in Chinese cattle

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ABSTRACT. Somatostatins play a crucial role in the regulation of growth and development in vertebrates, especially muscle growth. We assessed the association of somatostatin gene polymorphisms with growth traits by PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) and DNA sequencing methods in 694 individuals from six Chinese cattle breeds. A novel single nucleotide polymorphism, G126A, was detected, and significant associations were found with body length, body height, hip width, heart girth, and hucklebone width index. Polymorphism of the somatostatin gene was found to be highly associated with growth traits in the Qinchuan breed at various ages. Gene frequency analysis showed significant differences among the breeds. Individuals with genotype AA had significantly lower body height, body length, hip width, and hucklebone width values compared to AG at 1.5 years old, and had significantly lower hip width, body length and hucklebone width compared to AG at 2 years old. At 2.5 years old, populations with genotype AA had significantly lower body length, hip width

and hucklebone width than AG individuals, with the exception of the Luxi breed, in which two genotypes were found. The Luxi and Ximentaer crossbreed had the lowest frequency of the G allele, while the highest G allele frequencies were found in the Luxi breed.

Key words: Cattle; SST gene; PCR-SSCP; Growth traits; SNPs

INTRODUCTION

The somatostatin (SST) is a cyclic tetradecapeptide, a member of the transforming growth factor (TGF-β) family, but not completely belonging to the current TGF-β subfamily because of considerable differences, such as skeletal shape and morphogenetic protein and statin properties. SST is one of the most important genes in regulating animal growth and development, where it plays a negative role in animal growth regulation by inhibiting the release of growth hormone (GH), hindering the animal's growth. SST was originally isolated from the hypothalamus of sheep as a 14-amino acid peptide in 1973 by Brazeau. Its relative molecular mass is 1637, including different forms of peptides, namely SST, SST14, SST28, and so on. SST is produced by neuroendocrine, inflammatory, and immune cells, in response to ions, growth factors, neuropeptides, and a series of cytokines. The peptide is released in large amounts from storage pools of secretory cells, or in some activated immune and inflammatory cells (Reichlin, 1983).

So far, six SST gene candidates were identified from the zebrafish genome database. Among them, SST1, SST2, SST3, and SST4 have been previously reported in vertebrates (Devos et al., 2002; Tostivint et al., 2008). Somatostatin 5 (SST5) and somatostatin 6 (SST6) are newly discovered (Liu et al., 2010). SST1 has been generally described in all vertebrates from agnathans to mammals (Andrews et al., 1988). All of these SSTs accomplish their diverse number of regulatory functions by interacting with high affinity somatostatin receptors (SSTR) in the plasma membrane (Bruno et al., 1992).

Along with the further study of the features of SST, a fairly large number of functions emerged. Due to its specialty of acting as an endogenous inhibitory regulator of the secretory and proliferative responses of target cells widely distributed in the brain and periphery and of hormone secretion in the pituitary gland, SST have an immeasurable effect on coordinating various aspects of growth, development and metabolism of animals through its inhibition of hormone secretions and cell proliferation and affecting nutrient absorption in the alimentary canal (Yamada et al., 1993; Kubota et al., 1994).

It is well known that growth in vertebrates is mainly controlled by GH (Planas et al., 2000; Butler and Le Roith, 2001). GH is produced and secreted in a manner reflective of the balance of stimulatory and inhibitory signals received by somatotrophs (Canosa et al., 2007). Evidence suggests that SST are the primary inhibitors of GH release (Very and Sheridan, 2002). Considering the vital function of GH in bovine growth and development and the inhibitory effect of SST on GH, as well as the rising living standard in our country, beef supply has become more and more insufficient and many weaknesses, such as slow growth, small body configuration and little amount of meat, exist in our cattle breeds, especially in the Qinchuan breed. The polymorphism of SST was detected and the correlation with growth traits was analyzed to provide preliminary scientific data for improving

the establishment of meat production performance by a molecular screening system and the breeding of new beef cattle strains.

MATERIAL AND METHODS

Sample collection data source

Blood samples were collected from 694 unrelated Chinese cattle from Shaanxi, Henan and Shandong provinces in China belonging to the following six breeds: Qinchuan, QC (N = 389); Luxi, LX (N = 64); Nanyang, NY (N = 48); Jiaxian, JX (N = 73); Xianan, XN (N = 67); Luxi and Ximentaer crossbreed, LXC (N = 53). Many records of growth traits and body size including body length (BL), body height (BH), waist height (WH), rump length (RL), hip width (HIP), chest depth (CD), chest circumference (CC), and hucklebone width (HW) of each individual from the different breeds mentioned above were collected for statistical analysis. Blood samples were taken back to the laboratory and stored at -80°C.

DNA extraction

Genomic DNA was isolated from blood samples following the traditional phenol-chloroform extraction method and dissolved in sterile water at a concentration of 100 ng/mL and stored at -20°C, which was used for polymerase chain reaction-single-strand conformational polymorphism (PCR-SSCP) assays.

Primer design and PCR amplification

PCR primers (F 5'-GTTTGACCAACCGCACTC-3' and R 5'-AGTTAGGGGATTCG GGTG-3') were designed from the sequences reported (GenBank accession No. NC_007299) in order to amplify a 234-bp fragment of the single exon of SST gene spanning the G126A substitution. A 15-µL reaction volume included 1 µL (50 ng/µL) template, 7.5 µL 2X *Taq* PCR MasterMix, 5.9 µL ddH_2O, and 0.3 µL of each primer (10 µmol/µL), performed in a Tpersonal thermocycler (Biometra) according to the following conditions: 95°C for 5 min (preliminary denaturation) followed by 31 cycles of 94°C for 30 s (denaturation), primer annealing at 57°C for 35 s, and 72°C for 35 s, and a final extension at 72°C for 10 min. PCR products were then electrophoresed on 1.5% agarose gels using 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA), containing 200 ng/mL ethidium bromide to detect the product.

SSCP and DNA sequencing

To screen for polymorphism in the fragments analyzed, PCR amplicons were subjected to SSCP analysis. Four microliters of the PCR product was mixed with 9 µL loading dye (98% formamide, 10 mM EDTA, 0.025% bromophenol blue, and 0.025% xylene-cyanol) after denaturing at 98°C for 10 min; the mixture was immediately chilled on ice for 10 min. Afterward, the mixture was loaded on a 10% acrylamide/bisacrylamide (29:1) gel and electrophoresed at 110 V for 14-20 h in 1X TBE buffer. Afterward, the polyacrylamide gel was

silver stained with 0.1% $AgNO_3$ and revealed with 2% NaOH (Lan et al., 2007). According to the PCR-SSCP band patterns that were visualized on the gels with the help of visual light, individual genotypes were defined (Qu et al., 2005). Amplicons derived from the three allele standards were included on each gel for reference (Byun et al., 2008).

Representative PCR products corresponding to different mutation types that stand for homozygous individuals of different genotypes were chosen for DNA sequencing using primer 1 previously designed. Forward and reverse reactions were both performed to rule out false positives. With the DNAStar program, the sequencing results were blasted to specifically recognize the polymorphism type and location.

Statistical analysis

The genotypic and allelic frequencies in six cattle breeds were calculated by the χ^2 test. Associations between genotypes of SNP1 (simple nucleotide polymorphism) of the SST gene and eight growth indices (BL, BH, WH, RL, HIP, CD, CC, and HW) and genetic effects were analyzed using the general linear model (GLM) procedure in Statistical Program for Social Sciences (SPSS), version 17.0, package, respectively. The following model were used:

$$Y_{ij} = \mu + A_i + G_j + (A + G)_{ij} + E_{ij},$$

where Y_{ij} is the trait measured on cattle, μ is the population mean, A_i is the fixed effect of age, G_j is the fixed effect of genotype, $(AG)_{ij}$ is the interaction between the age and the genotype, and E_{ij} is the random error. Significant differences between least-square means of different genotypes were determined using the Duncan multiple-range test, given that the trait was excluded from the model if its effect was not significant ($P > 0.05$). The values are reported as least square means and standard error of the means. P values of 0.05 were considered to be statistically significant (Lan et al., 2007).

RESULTS

PCR-SSCP analysis

The PCR-SSCP method was adopted for the identification of nucleotide sequence polymorphism of the bovine SST gene. The PCR products obtained at the most suitable temperature were detected, and the amplification result was satisfactory (Figure 1). The target fragment of the gene was amplified and denatured, and the polymorphism was found in the region of exon 1 (locus A) by polyacrylamide gel electrophoresis (Figure 2). In the region of exon 2, no polymorphisms were detected under all the possible electrophoretic conditions (Figure 3). By analyzing the PCR products of primer 1, three kinds of genotypes were detected in locus A by PCR-SSCP in the Luxi breed (Table 1). However, only two kinds of genotypes were found in the other five cattle breeds. This variant was confirmed by sequencing the PCR products from each genotype of chosen individual. The homozygote, consistent with the sequence of GenBank accession No. NC_007299, was named the AA genotype, another homozygote was named the GG genotype, and the heterozygote was named the AG genotype (Figure 4).

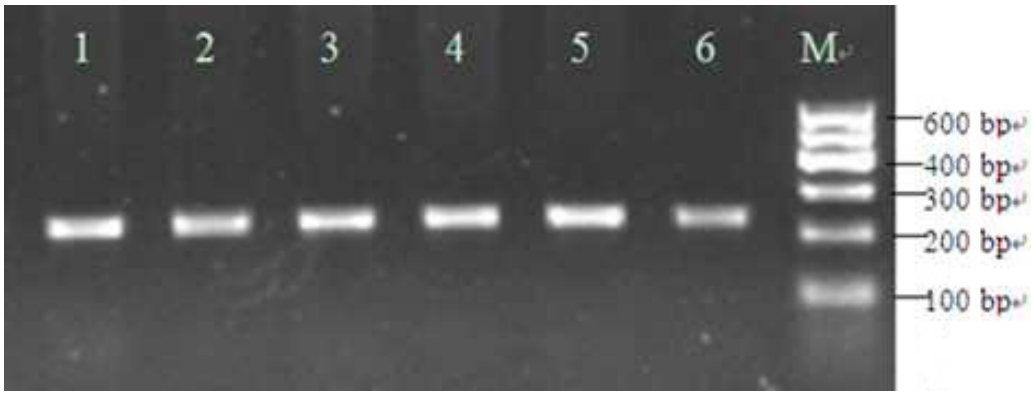

Figure 1. PCR amplification. *Lanes 1- 6*: PCR products; *lane 7* = marker 1.

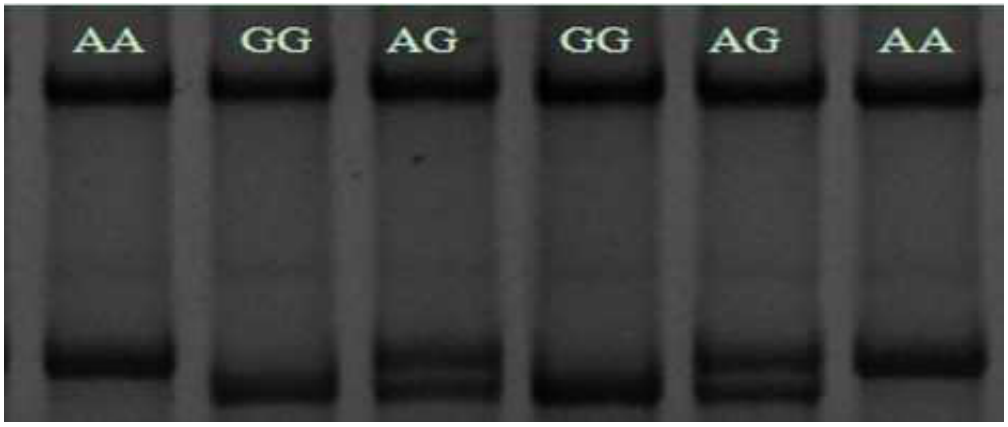

Figure 2. SST PCR-SSCP defined from genomic DNA amplifications on a silver-stained gel. The observed SSCP alleles are indicated at the bottom.

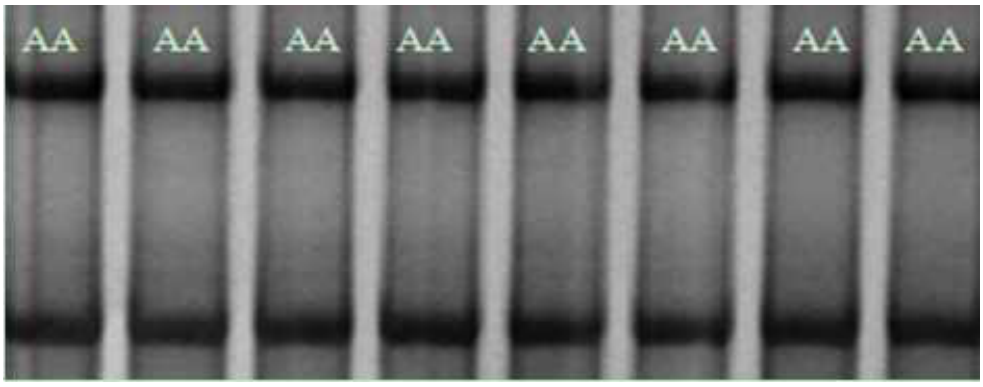

Figure 3. The PCR-SSCP of SST exon 2; no polymorphisms were detected.

Table 1. Genotype distribution and haplotype frequencies of SST exon 1 locus A.

Breeds	Observed genotypes			Total	Haplotype frequencies	
	AA	AG	GG		A	G
QC	274	115	0	389	0.8522	0.1478
LX	34	25	5	64	0.7266	0.2734
NY	37	11	0	48	0.8854	0.1146
JX	60	13	0	73	0.9110	0.0890
XN	56	11	0	67	0.9179	0.0821
LXC	47	6	0	53	0.9434	0.0566

QC = Qinchuan cattle; LX = Luxi cattle; NY = Nanyang cattle; JX = Jiaxian cattle; XN = Xianan cattle; LXC = Luxi and Ximentaer crossbred cattle.

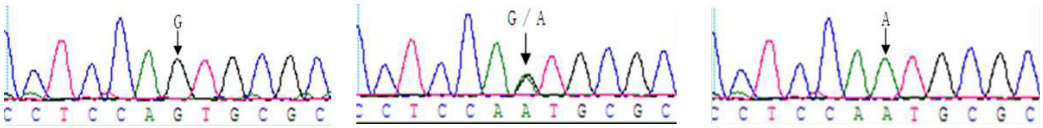

Figure 4. Sequences of three genotypes of SNP1 locus A (primer 1). **A.** Sequence of the AA genotype of SNP1 locus A (primer 1); **B.** Sequence of the AG genotype of SNP1 locus A (primer 1); **C.** Sequence of the GG genotype of SNP1 locus A (primer 1).

Genotypic and allelic frequencies

Each experimental individual was statistically analyzed with respect to the SST genotype. The frequency of genotype AG was low in the six breeds (QX, 0.2956; LX, 0.3906; NY, 0.2292; JX, 0.1781; XN, 0.1642; LXC, 0.1132), and genotype GG was only found in the Luxi breed. Accordingly, the frequency of allele G was very low in the six breeds (0.0566-0.2734) (Table 1).

The population genetic indices (gene homozygosity, gene heterozygosity (He), effective allele number (Ne), and polymorphism information content (PIC)) were calculated by the χ^2 test (Table 2). The values of PIC and He of the Luxi breed in the loci were higher than that of other populations, which implied that the polymorphism and genetic variation of the Luxi breed were higher than that of other populations, showing higher polymorphism. Gene homozygosity varied from 0.6027 (LX) to 0.8932 (LXC), and Ne ranged from 1.1196 (LXC) to 1.6593 (LX). The maximum and minimum PIC values were 0.3184 (LX) and 0.1011 (LXC). This showed that it was relatively stable within the Chinese bovine SST gene in the populations analyzed except for the Luxi breed, indicating that more relevant study should be conducted on the Luxi breed.

Table 2. Genetic diversity at SST gene exon 1 locus.

Breed	Homozygosity	Heterozygosity	Effective allele number	Polymorphic information content (PIC)
QC	0.7481	0.2519	1.3368	0.2202
LX	0.6027	0.3973	1.6593	0.3184
NY	0.7971	0.2029	1.2546	0.1823
JX	0.8378	0.1622	1.1936	0.1491
XN	0.8493	0.1507	1.1774	0.1393
LXC	0.8932	0.1068	1.1196	0.1011

PIC >0.5 means high diversity, 0.25 < PIC < 0.5 means moderate diversity, PIC <0.25 means low diversity. QC = Qinchuan cattle; LX = Luxi cattle; NY = Nanyang cattle; JX = Jiaxian cattle; XN = Xianan cattle; LXC = Luxi and Ximentaer crossbred cattle.

Correlation between SST gene polymorphisms and growth performance

With the help of GLM estimation, the association analysis of the variant loci of SST gene of the Qinchuan breed and the growth indices was carried out (Table 3). Statistical differences were found in body length, body height, hip width, heart girth, and hucklebone width index of Qinchuan cattle at 1.5, 2, 2.5 years old. Multiple comparisons indicated that individuals with genotype AG had significantly higher values than those of individuals with genotype AA in body length, hip width, and hucklebone width at 1.5 and 2.5 years old ($P < 0.05$). Individuals with genotype AG had significantly higher values than those of individuals with genotype AA in chest circumference at 2 years old, while for the body length and hucklebone width, the difference was very significant ($P < 0.01$), which was the same as the body height index of 1.5-year-old populations, respectively. No statistically significant differences were observed between the AG and AA genotypes in waist height, rump length and chest depth indices of Qinchuan cattle at various age stages.

Table 3. Association of genotypes of SNP1 least-square means and standard errors (means ± SD) for the SST gene exon 1.

Age	Trait (cm)	AG	AA	P
1.5 years old	Body length	128.50 ± 4.198[a]	117.15 ± 2.852[b]	0.039
	Body height	118.00 ± 1.970[a]	108.85 ± 1.338[c]	0.001
	Waist height	122.33 ± 5.285	118.85 ± 3.590	0.592
	Rump length	40.00 ± 1.129	39.00 ± 0.767	0.474
	Hip width	39.00 ± 1.450[a]	35.23 ± 0.985[b]	0.046
	Chest depth	63.17 ± 2.003	58.77 ± 1.361	0.087
	Chest circumference	158.17 ± 5.096	147.00 ± 3.462	0.088
	Hucklebone width	22.33 ± 1.001[a]	18.92 ± 0.680[b]	0.012
2 years old	Body length	134.63 ± 2.472[a]	123.25 ± 2.331[c]	0.002
	Body height	120.13 ± 1.656	118.19 ± 1.561	0.402
	Waist height	123.88 ± 1.423	123.75 ± 1.342	0.949
	Rump length	43.66 ± 1.113	41.22 ± 1.050	0.121
	Hip width	42.78 ± 1.163[a]	38.72 ± 1.096[b]	0.016
	Chest depth	63.72 ± 1.284	63.89 ± 1.210	0.924
	Chest circumference	169.59 ± 3.198[a]	157.61 ± 3.016[b]	0.01
	Hucklebone width	26.41 ± 1.057[a]	21.61 ± 0.996[c]	0.002
2.5 years old	Body length	133.57 ± 2.856[a]	125.80 ± 1.951[b]	0.036
	Body height	119.43 ± 2.188	118.13 ± 1.494	0.630
	Waist height	122.57 ± 2.013	123.53 ± 1.375	0.697
	Rump length	43.93 ± 1.277	41.10 ± 0.872	0.082
	Hip width	43.93 ± 1.400[a]	40.33 ± 0.957[b]	0.047
	Chest depth	65.64 ± 1.544	64.03 ± 1.054	0.399
	Chest circumference	173.29 ± 4.196	163.00 ± 2.867	0.057
	Hucklebone width	27.86 ± 1.347[a]	24.33 ± 0.920[b]	0.043

[a,b]Different superscript letters in the same row indicate significant difference ($P < 0.05$). [a,c]Different superscript letters in the same row indicate highly significant difference ($P < 0.01$).

DISCUSSION

Up to now, there were very few reports about SST gene SNPs and their relationship with growth and development traits of animals, especially for cattle. Almost all research on SST focused on immune system, gain in weight and treatment for some neurologic and epidemical diseases (Patel, 1999; Kang et al., 2000). Our studies first examined SST as an candidate gene for bovine growth traits, using PCR-SSCP and DNA sequencing methods. One novel mutation, G126A, was found in exon 1, but no SNPs were found in exon 2. Some reports

indicated that the SST gene was considerably conservative. This is a perfect confirmation. Through statistical analysis, the AA genotype showed a higher frequency than AG in six cattle breeds, while GG showed the lowest one. This result indicated that AA was the dominant genotype and A was the dominant allele. This discrepancy may be due to the species selection.

The relationship between SNPs in exon 1 and growth indices of the Qinchuan breed from 694 individuals was analyzed. The data in Table 3 showed that the AG genotype of G126A could increase body length, hip width and hucklebone width of cattle at different age stages. It is worth noting that these indices are potent indicators of cattle meat production. We can take some scientific measures to maintain the heterozygous gene (AG genotype) that can increase body length, hip width and hucklebone width, so as to promote the production of meat and solve a hot potato of low per capita consumption of meat production in China or even in the world.

Based on these results and from a functional point of view, the interesting allelic variant reported in the present study, which is identified in the exon region of the SNP G/A gene, may influence regulatory expression elements and could probably be used as markers for growth index in a cattle breeding program, and SST is likely a key gene that is a determinant in the improvement of growth in cattle breeds. Moreover, PIC was a perfect index for identifying the allele polymorphism, and it was also a reflection of the level of gene mutation. Our study showed that in the Luxi breed, PIC of locus A was 0.3184, indicating a moderate level of polymorphism ($0.25 < PIC < 0.5$). Higher PIC demonstrates higher heterozygosis within an animal population, leading to more genetic alteration, which is beneficial for the genetic improvement of the correlated traits. Thus, it is possible that the Luxi breed may be a useful group, and it is worthy of further study.

Besides, because of the cost-efficient feature of PCR-SSCP for genotyping of the gene polymorphism at the population level and because it can provide an alternative to more sophisticated typing methods for small laboratories with limited resources, there is a growing interest in the detection and characterization of SNP markers for the purpose of paternity and identity confirmation (Heaton et al., 2002; Werner et al., 2004). The efficacy of polymorphisms depends on their information content. If the SST polymorphism is going to be tested in association studies in the population at large, it could be useful to include it in an SNP panel designed for the purposes mentioned above.

In conclusion, this study showed a new polymorphic locus (SNP1), which is meaningful for searching for more genetic markers. Through the correlation study on the polymorphism of SST and eight growth indices of Qinchuan cattle breed, the results indicated that this site was highly relevant to body length, hip width and hucklebone width features. Thus, adding cattle varieties, expanding samples and doing further correlation studies are still needed to accumulate quantitative molecular genetics data in studying the relationship between the SST gene and growth traits in cattle.

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Luxi cattle breeding center of Shandong province, and Snow dragon beef cattle breeding center of Liaoning province (P.R. China).

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