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Four novel polymorphisms of buffalo *INSIG2* gene are associated with milk production traits in Chinese buffaloes

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ABSTRACT

Insulin-induced genes (INSIGs), including *INSIG*1 and *INSIG*2, are important mediators that play a pivotal role in the lipid metabolism and could cause the retention of the SCAP/SREBP complex. Therefore, the objective of this study is to detect the single nucleotide polymorphisms (SNPs) of buffalo *INSIG*2 gene and evaluate their associations with milk production traits in Chinese buffaloes. A total of four SNPs (g.621272A > G, g.621364A > C, g.632543G > A, and g.632684C > T) were identified using DNA pooled sequencing, and the SNP genotyping for the identified SNPs was performed by using Matrix-assisted laser desorption/ionization time of flight mass spectrometry method from 264 individuals. The results showed that four SNPs were significantly associated with 305-day milk yield or protein percentage in Murrah and crossbred breeds ($P < 0.05$), but they had no significant effect on milk production traits in Nili-Ravi buffaloes ($P > 0.05$). Linkage disequilibrium (LD) analysis revealed that one haplotype block was successfully constructed, of which the diplotype H1H1 showed significant association with 305-day milk yield in Murrah buffaloes ($P < 0.05$). Our findings provide evidence that polymorphisms in buffalo *INSIG*2 gene are associated with milk production traits, and could be used as a candidate gene for marker-assisted selection in buffalo breeding program.

1. Introduction

Insulin-induced genes (INSIGs), including *INSIG*1 and *INSIG*2, are proteins that are involved in the sterol regulation of sterol regulation element-binding protein (SREBP) cleavage-activating protein (SCAP) and 3-hydroxy-3-methylgutary-coenzyme A reductase (HMG-CoA reductase), which plays an important role in the cholesterol metabolism, lipogenesis and glucose homeostasis [1]. As the SREBPs are important regulators for maintaining the balanced lipid content in the milk [2] and HMG-CoA reductase is a rate-limiting enzyme in the *de novo* synthesis of cholesterol [3,4], INSIGs serve as the regulators that mediate reaction of cholesterol synthesis in some animal tissues [5].

Initially, *INSIG*1 was found to be cloned from the regenerating liver [6]. Subsequently, the *INSIG2* gene was identified in a liver-specific transcript of rodent [7]. In turn, several studies showed that they shared 59% amino acids identity [1,8]. Interestingly, Han et al. [9] found that the *INSIG*1 mRNA expression level was very high in mouse mammary glands during the lactation cycle. Similar results for the *INSIG1* and *INSIG2* genes have also been found in cow [10] and buffalo [11]. These results implied that INSIGs protein might be involved in the regulation of milk synthesis in animals.

Recently, accumulating evidence indicated that the INSIGs polymorphisms were associated with the obesity [12], weight gain [13], hypercholesterolemia [14] and milk traits [15]. Given the key role of the INSIGs in the regulation of lipogenesis and cholesterol in animals, variants in the INSIGs might affect the production performance in animals. Indeed, some studies have revealed that INSIGs were highly correlated with lactation, growth, and carcass traits in cows [16]. Liu et al. [17] reported that four SNPs of *INSIG*1 were associated with growth and carcass traits in Qinchuan cattle. Rincon et al. [18] highlighted *INSIG2*-ss252452236 allele C was associated with milk fatty acids composition in Holstein cattle. However, no study on associations of *INSIG*2 gene with milk production traits in buffaloes has been reported. Therefore, our study is aimed to detect the single nucleotide polymorphisms (SNPs) of

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INSIG2 gene in the Murrah, Nili-Ravi and crossbred breeds and estimated the associations between these SNPs and milk production traits in the studied populations.

2. Materials and methods

2.1. Animals and phenotypic records

Three buffalo breeds with a total of 264 individuals were used to identify the SNPs of buffalo *INSIG2* gene, including Murrah (n = 55), Nili-Ravi (n = 63) and Crossbreds (n = 146; Murrah × Local, n = 80 and Nili-Ravi × Local, n = 66). All the selected buffaloes were kept at a farm belonged to the Buffalo Research Institute, Chinese Academy of Agricultural Sciences (BRI-CAAS). Phenotype data including 305-day milk yield, protein percentage, and fat percentage were collected by the BRI-CAAS in the period from Jan 2010 to Dec 2012.

Genomic DNA was isolated from the blood sample of each individual using TIANamp Blood DNA Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The DNA concentration and quantity were measured using the Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE) and 1.5% agarose gels, respectively.

2.2. SNP identification and genotyping

Fifth buffalo samples were randomly chosen to identify variants of *INSIG2* gene in buffalo. According to the buffalo *INSIG2* mRNA sequence (GenBank accession number KF975668.1), the whole *INSIG2* gene sequence was obtained using the BLAT tool [19]. All exons and their adjacent intronic sequences were used for selective amplification by the polymerase chain reaction (PCR). Nine pairs of primers (Table A.1) were designed to detect the SNPs of *INSIG2* gene using Primer Premier 5.0 software.

PCR amplification was performed in 50.0 μL reaction mixtures, containing 1.0 μL genomic DNA template (20.0–30.0 ng), 2.0 μL Primers mix (10 μM of each), 25.0 μL Premix™ LA Taq (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) and 22.0 μL double-distilled H_2O. PCR procedure was run in a Biometra PCR machine (Analytic Jena, GER) with the following procedure: 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 57–59 °C, and 45 s at 72 °C, and a final extension of 8 min at 72 °C. PCR products were sent to the company for sequencing (Invitrogen Life Technologies Corporation, Guangzhou, China). Searching sequence mutation was performed using the SeqMan software (DNASTAR Inc., Madison, WI).

The identified SNPs were then genotyped in 264 individuals using the iPLEX MassARRAY system (Sequenom's MassARRAY) by the company (Invitrogen Life Technologies Corporation, Guangzhou, China).

2.3. Statistical analyses

Allelic frequencies, genotypic frequencies, heterozygosity, polymorphism information content (PIC) and Hardy-Weinberg equilibrium (HWE) were calculated for each locus using R language with the genetics package [20]. The Haploview software Version 4.2 was used to estimate the linkage disequilibrium (LD) and haplotype frequencies of all SNPs [21].

Marker-trait association analysis was performed using General Linear Model (GLM) procedure by SAS V9.4 software (SAS Institute Inc., Cary, NC) with the following model: $Y_{ijklm} = \mu + Y_i + P_j + S_k + DIM_l + G_m + e_{ijklm}$, where Y_{ijklm} = trait observation; μ = overall mean, Y_i = fixed effect of the ith year, P_j = fixed effect of the jth age-parity classes, S_k = fixed effect of the kth year-season of calving; DIM_l = fixed effect of the lth stage of lactation (60 d groups: 1–60, 61–120, …≥361), G_m = fixed effect of the mth genotype, e_{ijklm} = random residual. Results on the *INSIG2* genotype effects were presented as least square means ± SE and Bonferroni correction for multiple F-testing was applied to the pairwise comparisons among different levels of fixed effects included in the model.

3. Results

3.1. SNPs identification and genotyping

In this study, we utilized the DNA pool sequencing to detect the sequence polymorphisms of buffalo *INSIG2* gene. A total of four SNPs (g.621272A > G, g.621364A > C, g.632543G > A, and g.632684C > T) were identified that were located in intron1 and intron2, respectively (Fig. 1A), which has been submitted to the Single Nucleotide Polymorphism (dbSNP) database (Accession No. ss# 1985401067, 1985401068, 1985401069, and 1985401070). In addition, Fig. 1B describes the genotyped plots of the four SNPs in 264 individuals from three buffalo breeds using Sequenom's MassARRAY method. The different genotypes could be exactly distinguished and the average genotyping call rate was 97.6%.

3.2. Genetic diversity

To investigate the genetic diversity of the studied population, the genotypic and allelic frequencies, heterozygosity and PIC were estimated and given in Table 1. The $\chi 2$-text results showed that the four SNPs in Murrah and Nili-Ravi breeds were in HWE ($P > 0.05$), whereas those in crossbreed deviated from HWE ($P < 0.05$ or $P < 0.01$), implying that both Murrah and Nili-Ravi buffalo breeds had undergone a low selection pressure. According to the classification of PIC, all the SNPs were intermediate polymorphism ($0.25 < PIC < 0.50$) in all the breeds. These results reflected that there was a low genetic diversity within the *INSIG2* in the populations.

3.3. Linkage disequilibrium analysis and haplotypes construction

To obtain more genetic information, we conducted the LD and haplotype analysis using HAPLOVIEW software. Herein, LD among the SNPs was estimated in the population according to Gabriel et al. [22], and the results showed that the D' value was ranged from 0.5118 to 1.000, which were in high linkage disequilibrium (Table A.2). Additionally, one haplotype block was successfully constructed with four SNPs and covered a region of about 11 kb in Murrah and crossbreed breeds (Fig. A.1). The haplotype frequencies are shown in Fig. 2. Of which, three major haplotypes were formed that accounted for 95.5%, 97.6%, and 98.6% individuals in Murrah, Nili-Ravi, and crossbred breeds, respectively.

3.4. Association analysis

Effects of the identified SNPs of buffalo *INSIG2* gene on milk production traits in Chinese buffaloes were calculated using a General Linear Model and the results were listed in Table 2. Four SNPs showed different degree of significant associations with 305-day milk yield or protein percentage in Murrah and crossbreed, respectively ($P < 0.01$ or $P < 0.05$). However, no SNPs locus was found to be associated with milk production traits in Nili-Ravi ($P > 0.05$) (no data were shown). For the Murrah buffalo, the buffaloes with AA genotype at g.621272A > G and g.621364A > C loci showed highest milk yield compared with the other genotypes after Bonferroni correction for multiple F-testing ($P < 0.05$). At the