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Molecular cloning, characterization and association analysis of the promoter region of the bovine *CDK6* gene

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ABSTRACT. Cyclin-dependent kinase 6 (CDK6) is a key element of D-type cyclin holoenzymes. It is involved in the regulation of the G1-phase of the cell cycle and is considered to be an important candidate gene for selection of body measurement traits through marker-assisted selection. We cloned the promoter sequence of this gene in bovines and found it to share high similarity with that of the human CDK6 promoter. A 2271-bp sequence upstream of the start codon in the bovine CDK6 5'-flanking sequence is rich in GC; it lacks consensus TATA or CAAT box, but it contains several MZF1 binding sites. Other potential cis-regulatory elements were found in the 5'-flanking region, including CdxA, SRY, p300, GATA-1, and deltaE. Allele frequencies were also analyzed in various cattle breeds (Qinchuan, Qinchuan improvement steers, Nanyang, Jiaxian red, Xia'nan, Luxi, Simmental and Luxi crossbred steers, and Xuelong) and association with a selected single nucleotide polymorphism (SNP) was calculated. The T-1075C SNP in the promoter was found to be significantly associated with body length and

heart girth. This SNP marker was found to be significantly associated with body length and the heart girth in 737 individuals. We conclude that this SNP of the CDK6 gene has potential as a genetic marker for important body traits in bovine reproduction and breeding.

Key words: Cattle; CDK6 gene; SNP polymorphism; Association analysis; Body measurement

INTRODUCTION

Cyclins and cyclin-dependent kinases (*CDKs*) are the principle positive regulators of the cell cycle. Among them D-type cyclins and their partners are the first cell cycle machinery reacting with extracellular signals (Yang et al., 2009). *CDK6* was identified as a novel kinase, and found to partner with the D-type cyclins and to possess pRb kinase activity *in vitro* and has since been understood to function solely as a pRb kinase in the regulation of the G1 phase of the cell cycle (Grossel and Hinds, 2006; Rowell and Wells, 2006). Meanwhile, in the human and mouse *CDK6* gene, promoter structure and activity in human *CDK6* have been reported, which play a role in differentiation of a variety of cell types (Thomas et al., 1999; Cram et al., 2001; Kohrt et al., 2009). Expression of *CDK6* in primary mouse astrocytes resulted in drastic changes in cellular morphology that correlated with changes in expression patterns of known markers of glial differentiation (Ericson et al., 2003). Also, the entry of mouse erythroleukemia cells into terminal differentiation was accompanied by a decline in the activity of *CDK6* whereas maintenance of *CDK6* activity blocked differentiation (Matushansky et al., 2003). Furthermore, osteoblast and osteoclast differentiation was inhibited by overexpression of *CDK6* (Ogasawara et al., 2004a,b). *CDK6* was also shown to inhibit myeloid terminal differentiation by preventing the developmentally important transcription factor, Run-X, from binding DNA (Fujimoto et al., 2007). *CDK6* was shown to be required for proper thymocyte development and tumorigenesis (Hu et al., 2009). Most recently, NANOG in embryonic stem cells, MEP50 in prostate cancer cells and Smad in osteoblast were shown to directly regulate *CDK6* transcription (Ogasawara et al., 2004a; Peng et al., 2008; Zhang et al., 2009). Additional evidence of *CDK6* function in developmental processes showed that *CDK6* expression was regulated by developmentally important miRNAs (Johnson et al., 2007; Lujambio et al., 2007; Silber et al., 2008). To our knowledge, there is so far no information available about polymorphisms of the bovine *CDK6* gene.

Based on the important roles of *CDK6* in chondrogenesis and osteocyte differentiation as determined in mouse and human, *CDK6* could be an attractive candidate gene for body measurement traits in bovine. The objective of this study was therefore to analyze molecular characterization and detect single nucleotide polymorphisms (SNPs) in bovine *CDK6* promoter and to explore their possible association with body measurement traits in *Bos taurus*.

MATERIAL AND METHODS

DNA samples and data collections

For the gene variants that were identified, allele frequencies were estimated on a restricted population composed of unrelated, randomly selected purebred and crossbred individuals representing 8 breeds including Qinchuan (QC, N = 69, Shaanxi province of China),

Qinchuan improvement steers (QI, N = 198, Shaanxi province of China), Nanyang (NY, N = 60, Henan province of China), Jiaxian red (JR, N = 74, Henan province of China), Xia'nan (XN, N = 78, Henan province of China), Luxi (LX, N = 75, Shandong province of China), Simmental and Luxi crossbred steers (SL, N = 55, Shandong province of China), Xuelong (XL; Angus crossed with descendant of male Japanese Black cattle and female Fuzhou cattle, N = 128, Liaoning province of China). Meanwhile, the following traits, body length (BL), withers height (WH), hip height (HH), rump length (RL), hip width (HW), chest depth (CD), heart girth (HG), and pin bone width (PBW) were measured (Gilbert et al., 1993). For each of the body measurements, the same traits were measured by the same person to minimize error.

DNA samples were extracted from leukocytes and tissue samples using standard phenol-chloroform protocol (Mullenbach et al., 1989).

Cloning and sequencing of bovine *CDK6* 5'-flanking regions

Compared with the available DNA sequences of human *CDK6*, the sequences of the 5'-flanking region of bovine *CDK6* available in GenBank are quite short, and the information is very limited. In order to obtain the sequences of the 5'-flanking region of bovine *CDK6*, human DNA sequences of *CDK6* (GenBank accession No. NC_000007) and bovine DNA sequences of *CDK6* (GenBank accession No. NC_007302) were compared with all sequences available in the Cow Genome Resources databases by using a BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast/). A *B. taurus* chromosome 4 genomic contig (GenBank accession No. NW_001494858.1) that contains partial genomic DNA sequences of bovine *CDK6* was selected to assemble the 5'-flanking region of bovine *CDK6*. A 2360-bp 5'-flanking DNA sequence was amplified by a pair of primers (Promoter-F and Promoter-R, Table 1) using the genomic DNA of Qinchuan cattle. Polymerase chain reaction (PCR) was conducted in 20 μL reaction mixtures containing 50 ng DNA templates, 10 pM of each primer, 0.20 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa, Dalian, China). The PCR procedure was: 95°C for 5 min followed by 34 cycles at 94°C for 30 s, 64°C annealing for 30 s, and 72°C for 120 s, and a final extension at 72°C for 10 min. The products were purified with a Wizard Prep PCR purification kit (Shanghai Bioasia Biotechnology, China) and sequenced (Beijing Aolaibo Biotechnology, P.R. China; Applied Biosystems 3730xl DNA sequencer, Foster City, CA, USA). Moreover, the PCR products were analyzed using Cattle dbSNP (http://www.ncbi.nlm.nih.gov/snp/limits), Transcription Element Search System (http://www.cbil.upenn.edu/cgi-bin/tess/tess), Promoter Scan (http://www.bimas.cit.nih.gov/molbio/proscan), and the NSITE program (Softberry, http://linux1.softberry.com/berry.phtml?topic=nsite&group=programs&subgroup=promoter).

Table 1. Primer data from experiments.

Primer name	Primer sequence (5'-3')	Binding region	PCR (Tm) (°C)	Size (bp)
Promoter-F	ATGGGGTCAAGTCACATTCAGC	Promoter	64	2360
Promoter-R	GCCTTGAACACCTTCCCGTAG	Exon 1		
SNP-F	GGTTCGGTAGGCGACTTCAGC	Promoter	65	82
SNP-R	TCGGGAGTGGGTGAGAGATGC	Promoter		

PCR = polymerase chain reaction; Tm = melting temperature; SNP = single nucleotide polymorphism; F = forward; R = reverse.

Polymorphism and genotyping of the *Pvu*II *CDK6* allele by ACRS-PCR-RFLP

Genetic polymorphisms in *CDK6* were identified by sequencing the 2360 PCR products and comparing them with the genomic contig (GenBank accession No. NW_001494858.1). An SNP -1075 (T/C) in bovine *CDK6* 5'-flanking regions was chosen for further analysis. Using SNP primers (SNP-F and SNP-R; Table 1), PCR was performed on samples from 737 individuals. PCR was done in 20 µL reaction mixtures containing 50 ng DNA templates, 10 pM of each primer, 0.20 mM dNTP, 2.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (TaKaRa). The PCR procedure was: 95°C for 5 min followed by 26 cycles at 94°C for 30 s, 65°C annealing for 30 s, and 72°C for 20 s, and a final extension at 72°C for 5 min. Allele frequencies were analyzed by genotyping the site for 737 individuals. Association analysis between genotypes and body measurement traits was carried out on our resource population. The amplification-created restriction site-PCR (ACRS-PCR) method, as described previously (Eiken et al., 1991; Chang et al., 1992a,b; Nafa et al., 1996), was employed to genotype the polymorphic sites. By using the restriction fragment length polymorphism (RFLP) technique, the products were analyzed on agarose gels and stained with ethidium bromide to assess size and quality. In addition, aliquots of 20 µL 82-bp PCR products were digested with 10 U *Pvu*II (MBI, Fermentas) at 37°C for 5 h following supplier instructions. The digested products were detected by electrophoresis on 4.0% agarose gel stained with ethidium bromide.

Statistical analysis

The following items were statistically analyzed according to previous approaches (Nei and Roychoudhury, 1974; Nei and Li, 1979; Liu et al., 2010), including genotypic frequencies, allelic frequencies, Hardy-Weinberg equilibrium, gene homozygosity, gene heterozygosity, effective allele numbers, and polymorphism information content (PIC). The association between SNP marker genotypes of the *CDK6* gene and records of body measurement traits (BL, WH, HH, RL, HW, CD, HG, and PBW) was analyzed by the least-squares method as applied in the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA), and according to the following statistical linear model:

$$Y_{ijkl} = \mu + G_i + S_j + BF_k + Ma_l + \varepsilon_{ijkl}, \quad \text{(Equation 1)}$$

where Y_{ijkl} is the observed body measurement trait, μ the overall mean for each trait, G_i the genotype effect, S_j the fixed effect of sex, BF_k the fixed effect of breed and farm, Ma_l the regression variable for measure age, and ε_{ijkl} the random environment effect.

RESULTS

Characterization of the bovine *CDK6* gene promoter

The 2360-bp fragment sequence, including a 2271-bp fragment upstream from the start codon and a 89-bp fragment of exon 1 in the bovine *CDK6* gene, was amplified (Figure 1A). A comparison with the human *CDK6* 5'-flanking sequence demonstrated a high degree of homology with approximately 84% nucleotide identity (Figure 2). Sequence analysis indicated several interesting features: as with the corresponding human sequence, no TATA box or CAAT box was found in the bovine sequence. Promoter prediction analysis indicated that they shared several confirmed binding sites for transcriptional factor, including CdxA, SRY, p300, GATA-1, MZF1, and deltaE,

and some of them were even located in identical positions. However, we also predicted four binding sites (Ik-2, Ik-1, CREB, and NF-E2) in the bovine sequence not found in human. Interestingly, we predicted a transcriptional start site (GTAAAGCTAGACCGATCTCCGGGG) of the bovine *CDK6* gene, which differed from the confirmed transcriptional start site (GAGAAAGCGAGACC GAACTCCGGGG) (Cram et al., 2001) by only four nucleotides (Figure 2).

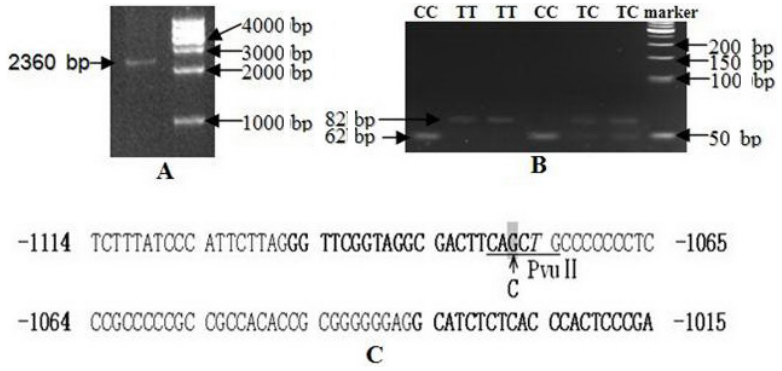

Figure 1. Information on cloning and amplification-created restriction sites for the bovine *CDK6* T-1075C locus. **A.** PCR products of the promoter region of the bovine *CDK6* gene. **B.** Agarose gel electrophoresis of *Pvu*II ACRS-PCR-RFLP at the bovine *CDK6* locus. TT genotype demonstrates one fragment (82 bp), TC genotype shows three fragments (82, 62, and 20 bp) and CC genotype shows two fragments (62 and 20 bp). The 20-bp fragment is not visible on 4.0% agarose gel electrophoresis, 82- and 62-bp fragments can classify the different genotypes (TT, TC and CC) exactly. **C.** The 82-bp sequence information of ACRS-PCR products. The ACRS-PCR-RFLP primers are shown in boldface, the *Pvu*II restriction site (CAG^CTG) with underline was created, instead of the "C" of the primitive sequence.

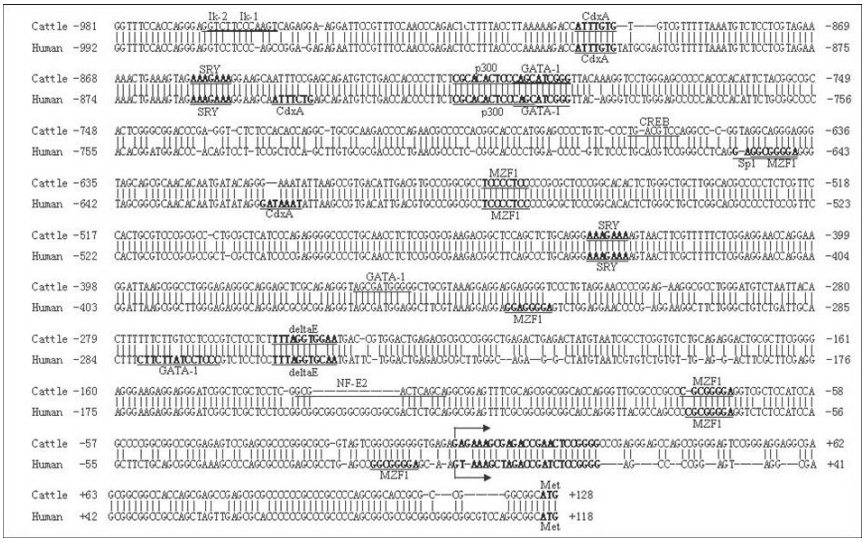

Figure 2. Comparison analysis between bovine and human *CDK6* proximal promoter regions. There were no TATA or CAAT boxes in the bovine and human *CDK6* gene. Arrows represent the transcriptional start site of the bovine *CDK6* gene, which was nearly identical to those of the human gene; potential transcription factor binding sites were predicted and indicated with underline, the sites only shared by cattle are not shown in boldface, and the start codon ATG is shown in boldface.

Polymorphism and sequencing of the *CDK6* gene

Compared with the genomic contig (GenBank accession No. NW_001494858.1), an SNP, named T-1075C, from a 2360-bp product of the *CDK6* gene promoter, was found. The T-1075C mutation, conformed to rs42524795 in Cow SNP Blast databases, was also observed at position -1075 upstream from the predicted transcription start site. Next, the *Pvu*II restriction site (CAG^CTG) was created, instead of the "C" of the primitive sequence (Figure 1C).

For the T-1075C SNP in the populations that we analyzed in this study, three size variants of restriction fragments were identified, namely, 82, 62 and 20 bp. Subsequently, we analyzed the localization of migration bands of the restriction fragments and discovered three genotypes of "mutation T>C". Our data in Figure 1B shows that genotype TT represents the occurrence of one band of 82 bp, genotype TC represents three restriction fragment bands of 82, 62 and 20 bp, and genotype CC represents two bands of 62 and 20 bp.

Genetic variation in different breeds and association analysis

We further analyzed the genotype of unrelated animals from 8 different bovine breeds, including (QC, QI, NY, JR, XN, LX, SL, and XL). Genotypic frequencies, allelic frequencies and Hardy-Weinberg equilibriums of the SNP in different populations were shown in Table 2. Moreover, allele frequencies of the SNP were investigated and performed by the χ^2 test in all bovine populations of our study (Table 2). The data shown here demonstrate that the range of frequencies of *CDK6*-T and -C allele was from 0.3590 to 0.4932 and from 0.5068 to 0.6410, respectively, among the 8 different subpopulations, and there was significant difference in the allelic frequency among all populations in the SNPs except the LX population ($P < 0.05$). Furthermore, frequencies of T/C alleles were 0.4376/0.5624 in the total population, which was also not in Hardy-Weinberg equilibrium ($P < 0.05$). The frequencies of the TT genotype were the lowest in three genotypes in all populations (Table 2). In addition, gene heterozygosity, effective allele numbers and PIC of the bovine *CDK6* locus in 8 populations varied from 0.4602 to 0.4999, 1.8526 to 1.9996, 0.3543 to 0.3750, and they were 0.4922, 1.9693 and 0.3711 in the total population, respectively (Table 3).

Table 2. Genotype frequencies (%) at the *CDK6* gene for the T-1075C SNP in bovine populations.

Breeds	Observed genotypes (number)			Total	Allelic frequencies		χ^2 (HW*)	P value (HW*)
	TT	TC	CC		T	C		
QC	0.0290 (2)	0.7971 (55)	0.1739 (12)	69	0.4275	0.5725	27.25	0.0000
QI	0.0202 (4)	0.8990 (178)	0.0808 (16)	198	0.4697	0.5303	128.2	0.0000
NY	0.0000 (0)	0.7667 (46)	0.2333 (14)	60	0.3833	0.6167	23.18	0.0000
JR	0.1081 (8)	0.7703 (57)	0.1216 (9)	74	0.4932	0.5068	21.64	0.0000
XN	0.0000 (0)	0.7179 (56)	0.2821 (22)	78	0.3590	0.6410	24.46	0.0000
LX	0.1333 (10)	0.5867 (44)	0.2800 (21)	75	0.4267	0.5733	2.97	0.2261
SL	0.0000 (0)	0.8727 (48)	0.1273 (7)	55	0.4364	0.5636	32.97	0.0000
XL	0.1406 (18)	0.6016 (77)	0.2578 (33)	128	0.4414	0.5586	6.19	0.0453
Total	0.0570 (42)	0.7612 (561)	0.1818 (134)	737	0.4376	0.5624	220.1	0.0000

HW = Hardy-Weinberg equilibrium; QC = Qinchuan; QI = Qinchuan improvement steers; NY = Nanyang; JR = Jiaxian red; XN = Xia'nan; LX = Luxi; SL = Simmental and Luxi crossbred steers; XL = Xuelong.

Table 3. Population genetic indexes at the *CDK6* T-1075C locus in bovine populations.

Breeds	Gene homozygosity	Gene heterozygosity	Effective allele numbers	PIC
QC	0.5105	0.4895	1.9589	0.3697
QI	0.5018	0.4982	1.9927	0.3741
NY	0.5272	0.4728	1.8967	0.3610
JR	0.5001	0.4999	1.9996	0.3750
XN	0.5398	0.4602	1.8526	0.3543
LX	0.5108	0.4892	1.9579	0.3696
SL	0.5081	0.4919	1.9681	0.3709
XL	0.5069	0.4931	1.9729	0.3715
Total	0.5078	0.4922	1.9693	0.3711

PIC = polymorphism information content. For breed abbreviations, see legend to Table 2.

Meanwhile, 8 body measurement traits were analyzed by comparing the genotypes of 737 individuals and their phenotypic data. The results of association analysis of the gene-specific SNP marker are shown in Table 4. At the T-1075C SNP marker, there were significant effects on BL ($P = 0.0091$) and HG ($P = 0.0009$) in the total population. Animals with the TT genotype had lower mean values for BL than those with TC and CC genotypes ($P < 0.05$). In the eight populations, there are significant effects on some body measurement traits, but the data are not shown.

Table 4. Association analysis of T-1075C single nucleotide polymorphism (SNP) genotypes with body measurement traits at the bovine *CDK6* gene.

SNP	Genotypes	Traits (cm)							
		BL	WH	HH	RL	HW	CD	HG	PBW
Total	TT	143.02 ± 2.50^{a}	131.59 ± 1.88	136.75 ± 1.69	44.26 ± 0.86	48.27 ± 1.20	71.61 ± 1.10	244.31 ± 57.37^{A}	23.10 ± 0.86
	TC	146.81 ± 0.54^{b}	133.07 ± 0.41	145.99 ± 3.03	44.04 ± 0.25	48.66 ± 0.27	72.79 ± 0.32	191.06 ± 0.99^{B}	24.85 ± 0.24
	CC	148.38 ± 0.98^{B}	132.80 ± 0.75	139.87 ± 0.81	44.37 ± 0.44	47.83 ± 0.62	72.64 ± 0.62	185.78 ± 2.23^{B}	24.29 ± 0.41
P		0.0091	0.5216	0.4511	0.8081	0.3631	0.564	0.0009	0.0631

Data are reported as means ± SEM. BL = body length; WH = withers height; HH = hip height; RL = rump length; HW = hip width; CD = chest depth; HG = heart girth; PBW = pin bone width. ^{a,b}Means with different lower case superscript letters were significantly different ($P < 0.05$). ^{A,B}Means with different capital superscript letters were significantly different ($P < 0.01$).

DISCUSSION

A number of potential regulatory elements including CdxA, SRY, p300, GATA-1, Sp1, MZF1, and deltaE binding sites have been identified in the human *CDK6* promoter (Cram et al., 2001), many of which can be also observed in the bovine *CDK6* promoter. Among these, two SRY, two MZF1, CdxA, p300, GATA-1, and deltaE binding sites were found in the bovine *CDK6* 5'-upstream sequence, and several of them were conserved in human and cattle. The human *CDK6* promotor does not include the typical TATA box, but contains a relatively GC-rich region in the proximal region. In the bovine sequence, these sites were identical to those in the human sequence in our study. Therefore, they may play a similar regulation role in both porcine and human.

Gene heterozygosity and effective allele numbers tended toward the mean. These results clearly indicate that the heredity of these populations and the sites controlling body

measurement traits were all relatively stable. Furthermore, the occurrences in all populations except the LX population were not in Hardy-Weinberg equilibrium due to random genetic drift. Frequencies of the TT genotype were low in all populations, due to the occurrence of random genetic drift of allele T. Generally, PIC was classified into the following three types: low polymorphism (PIC value <0.25), median polymorphism (0.25 < PIC value < 0.5), and high polymorphism (PIC value >0.5) (Mateescu et al., 2005). According to this classification of PIC, all populations belong to the median polymorphism level. Therefore, our data indicate that the high frequency of the *CDK6*-C allele at the bovine *CDK6* locus could be used to characterize native Chinese cattle breeds.

Body measurement traits are affected by many factors, including genotype, sex, age, breed, herd, location, and other random environmental factors. However, we have established one new statistical model, in which three factors (breed, herd and location) were involved and then we employed the least-squares method in the GLM procedure using the SAS software to do the related analysis, and we did not find a significant difference ($P > 0.05$) (data not shown).

For the T-1075C SNP marker at the bovine *CDK6* gene, there were significant effects on BL and HG in 737 individuals (Table 4). We assumed that the mutation for T-1075C could have an important influence on many minor genes, which involve BL and HG in bovine. A number of studies have recently demonstrated that functional SNPs in the *CDK6* gene have a plausible biological role in height and other stature indexes in human (Weedon et al., 2008; Gudbjartsson et al., 2008; Lettre et al., 2008). Weedon et al. (2008) found an SNP (rs2282978, $P = 7.8 \times 10^{-23}$), associated with height and occurring in intron 4 of the human *CDK6* gene, which was associated with *CDK6* expression ($P = 1.0 \times 10^{-6}$). Also, the human *CDK6* gene provided further evidence of a link between normal growth and unregulated cell differentiation (Weedon et al., 2008). Meanwhile, Gudbjartsson et al. (2008) found that human *CDK6* could drive a significant excess of loci neighboring genes with the 'chromosome segregation' classification, and two SNPs (rs2282978 and rs11765954) in *CDK6* were identified suggesting an association in a genome-wide association scan for height. Using data from GWA studies, Lettre et al. (2008) identified an association with height for human *CDK6* loci (rs2040494, $P = 3.8 \times 10^{-7}$). Above all, although many studies focus on the association of *CDK6* gene variants with body measurement traits in human, no available data have been reported for bovine and other livestock. Therefore, based on these results of the genome-wide approach in human and according to the conformity of the conservation of biological evolution in different organisms, we applied the research results from human *CDK6* in analyzing polymorphism and genetic effect to the cattle *CDK6* gene locus. The new findings indicate that the T-1075C SNP of bovine *CDK6* is significantly associated with body length and heart girth (Table 4), which are consistent with the human data.

In summary, we analyzed characterization of the promoter region of the bovine *CDK6* gene, and identified one SNP and investigated its association in different bovine breed populations. Genotyping analysis and association analysis were performed on the T-1075C SNP, demonstrating that the SNP was significantly associated with body measurement traits in bovine. Therefore, further study will be necessary to use the SNP for marker-assisted selection in other breeds and larger populations. It is also significant to investigate whether the *CDK6* gene plays a role in the development of those traits and whether it is involved in linkage disequilibrium with other causative mutations.

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