ГЕНОМИКА.
ТРАНСКРИПТОМИКА

NOVEL MUTATION IN TGA STOP-CODON OF BOVINE *SIX6* GENE

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As a transcriptional regulatory gene of the *SIX* family, *SIX6* (also known as *OPTX2*, *SIX9*), probably affects pituitary development and secretion of hormones, suggesting that this gene is a potential candidate gene for studying association with growth trait in animals. Therefore, this study is first of all focused on detecting sequence variations in a bovine *SIX6* gene and on its effects on growth traits in 1087 cattle from five Chinese cattle breeds using DNA sequencing and HhaI-ACRS-PCR methods. Herein, a novel mutation (NC_007308: g 2015T > C) in the TGA stop-codon of a bovine *SIX6* gene was found, which leads to an ORF shift and extension of the encoded protein for four amino acids (Arg²²³-Gln²²⁴-Arg²²⁵-Val²²⁶). The frequency of allele "C" varied from 0.255 (Chinese Holsteins) to 0.614 (Hasake). They all were in the Hardy-Weinberg equilibrium except Jiaxian Red and Hasake cattle. Using amino acid sequence alignment and online prediction software, a new helix in the C-terminal domain of the mutated bovine *SIX6* protein was revealed, which possibly affects pituitary development and hormone secretion. So, relationship analysis between this polymorphism and growth traits in the Nanyang breed was carried out based on a proper linear model. Although no statistically significant associations were observed ($P > 0.05$), the presented work preliminarily demonstarted a novel mutation in the TGA stop-codon which extends the spectrum of genetic variations of the bovine *SIX6* gene and might be of interest in terms of its association with other biophysical and biochemical indexes.

Keywords: *SIX6* gene, stop-codon, mutation, ACRS-PCR, growth traits, cattle.

The pituitary gland regulates basic physiological functions, including growth, stress response, reproduction, metabolism and lactation. Recently, development mechanisms and the complex regulation network of the pituitary gland have been extensively and intensively studied in mice and humans, especially its involvement in the secretion of many hormones, such as ACTH (Adrenocorticotrophic hormone), TSH (Thyroid-stimulating hormone), GH (Growth hormone), PRL (Prolactin), LH (Luteotrophic hormone), FSH (Follicle-stimulating hormone) and so on [1, 2]. In order to further study functions and regulation mechanisms of the pituitary gland, POU, PAX, LIM, SIX, OAR, ELK, bZIP, PHD-finger, Engralled and hexapeptide-main homeobox transcription factors have been identified and studied in different species. They all have a homeodomain which is associated with DNA binding or protein interactions. So, sequence variations of the homeobox transcription factors above including SIX6 will possibly have a significant effect on the basic physiological functions of the pituitary gland [3, 4].

SIX6 (sine oculis homeobox homolog 6) is a novel homologue of *SIX* gene family of transcriptional regulatory genes in vertebrates, which encodes a highly diverged nuclear homeoprotein with two highly conserved motifs: a SIX domain (SD) that is involved in protein–protein interactions and a homeodomain (HD) that binds DNA

[5–8]. Human *SIX6* is 2567 bp in length and is split in two exons that are transcribed into a 1393-nucleotide-long mRNA. The *SIX6* homeoprotein which consists of an N-terminal (NT) peptide of 11 amino acids, a SIX domain (SD) of 115 amino acids, a homeodomain (HD) of 60 amino acids, and a C-terminal domain (CT) of 60 amino acids plays an important role in embryonic development, pituitary development, gene regulation, cell differentiation, neurogenesis and so on. Chromosomal mapping of *SIX6* revealed that it is closely linked to *SIX1* and *SIX4* in the human chromosome 14q22.3–q23 (a region that was found to be deleted in three individuals with bilateral, anophthalmia and pituitary anomalies), *SIX6* is also closely related to *SIX3* and is expressed in the developing and adult retina, and hypothalamic and pituitary regions [8, 9]. *SIX6* expression is largely confined to the hypothalamus during postnatal brain development. Deletion of the *SIX6* gene resulted in hypoplastic pituitary and retina [10–13]. *SIX6* haploinsufficiency was also found to be responsible for bilateral anophthalmia, the absence of the optic nerve and chiasma and pituitary abnormalities [14, 15]. Briefly, the human *SIX6* gene plays an important role in the early development of the pituitary gland and visual system and potentially is considered as a candidate gene for the defects, which also provide clues about the origin and evolution of the vertebrate *SIX* gene families. However, no mutation or deletion of the *SIX6* gene was revealed in the development of the pituitary gland in rumi-

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Table 1. Primer information of the bovine *SIX6* gene

Loci	Primer	Fragment, bp	T_m, °C	Note
P1	1F:5'-TCCTCCAATCCCTCCCG-3' 1R:5'-TGTAGCCTTGGTATGGTGACTG-3'	995	61	5'UTR/Exon1/Exon2/intron1/intron2 (73−1067)
P2	2F:5'-GCTGTGGCTCGAAGCTCATTAC-3' 2R:5'-CGTTCAAGGCTCGGAGGTCTAT-3'	339	60	Exon 2/Intron1 (518−856)
P3	3F:5'-GGGCTGACTGCTGGGCTTA-3' 3R:5'-CGCTGGGTTGCTTGGTCT-3'	384	65.5	Exon3/Intron2/3'UTR (1764−2147)
P4	4F:5'-TCCTCCAATCCCTCCCG-3' 4R:5'-CGTTCAAGGCTCGGAGGTCTAT-3'	784	60	5'UTR/Exon1/Exon2/intron1/intron2 (73−856)
P5	5F:5'-GCTGTGGCTCGAAGCTCATTAC-3' 5R:5'-TGTAGCCTTGGTATGGTGACTG-3'	550	58	Exon2/Intron1/Intron2 (518−1067)
P6	F3:5'-GGGCTGACTGCTGGGCTTA-3' Rnew:5'-GGGCAACTCAGATGTCACACT-CGCTGGC-3'	279	65.5	Exon3 (1764−2042)

nant animals. This gene could be an important candidate gene for association studies with growth trait in animals.

In order to further explore abundant candidate gene resources for the QTL loci among several quantitative traits and provide a solid theory on animal breeding, the aim of this study was to detect sequence variations of the bovine *SIX6* gene and to analyze its effects on growth traits in 1087 individuals from five Chinese cattle breeds (Qinchuan, Nanyang, Jiaxian, Jiaxian Red, Chinese Holsteins and Hasake), which will be used for developing the bovine industry in the MAS project, using DNA sequencing and ACRS (allele created restriction site)-PCR methods,

EXPERIMENTAL

Genomic DNA samples and animals sources. Genomic DNA samples were obtained from 1078 healthy female individuals belonging to five breeds: Qinchuan cattle (QC, $n = 236$) were from the protection region of Qinchuan cattle (Weinan city, Shaanxi Province, P.R. China), the breeding farm of Qinchuan cattle and the fineness breeding center of Qinchuan cattle (Fufeng county, Shaanxi Province, P.R. China), Jiaxian Red cattle (JX, $n = 435$) were from the breeding protection region of Jiaxian cattle (Jiaxian county, Henan Province, P.R. China), Nanyang cattle (NY, $n = 269$) were from the breeding center of Nanyang cattle (Nanyang city, Henan Province, P.R. China), Chinese Holsteins (CH, $n = 94$) were from the breeding farm of milk breed (in Xi'an City, Shaanxi province, P.R. China), and Hasake cattle (HA, $n = 44$) were from the breeding farm of beef breed (Yili city, Xinjiang province, P.R. China). Among them the QC, NY and JX were beef breeds. The Chinese Holsteins was a dairy breed and the Hasake was a foreign improved breed. DNA samples were extracted from leucocytes using the sal-chloroform extraction protocol [16].

Primer design and PCR conditions. Six pairs of primers were designed on the base of the whole sequence of the

bovine *SIX6* gene (GenBank acc. no. NC_007308) in order to amplify the coding and flanking regions using Primer V5.0 software (Table 1). The PCR amplification reaction was carried out in a total volume of 25 μL in the presence of 50 ng of bovine genomic DNA as the template, 0.5 μM of each primer, 200 μM dNTPs (dATP, dTTP, dCTP and dGTP), 1 × buffer [including 1.5 mM MgCl$_2$] and 0.625 U Taq DNA polymerase ("MBI Fermentas"). The cycling protocol was 4 min at 94°C, 34 cycles of 94°C for 30 s, annealing at 65.5°C for 30 s, 72°C for 20 s, with a final extension at 72°C for 10 min.

Identification and genotyping of SNP. DNA pool mixture sequencing (ABI 377 DNA Analyzer) and alignment of nucleotide sequences in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) database for homologous sequences showed a novel mutation (NC_007308:g.2015T > > C) in the TGA stop-codon locus within the bovine *SIX6* gene. In order to detect this SNP the ACRS (allele created restriction site) method was used. The new reverse primer (P$_3$R$_{new}$: 5'-GGGCAACTCAGATGTCACACTCGCT-G G C-3') was designed by a single nucleotide mismatch, where the actual nucleotide "T" was replaced by a "C". So, a HhaI restriction site (GCG C) was created in the PCR products from g.2015C individuals, whereas PCR products from the g.2015T individuals lacked this site. PCR amplification was carried out using the forward PCR primer (P$_3$F3) described above and the new reverse PCR primer (P$_3$R$_{new}$) in order to obtain PCR products from all bovine individuals. Then 10 μL aliquots of PCR products were digested with 10 U HhaI ("Promega Corporation", USA) according to the supplier's directions for buffer conditions. The digested products were detected by 10.0% polyacrylamide gel electrophoresis (PAGE) (80 × 73 × × 0.75 mm) in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM Na$_2$EDTA) at a constant voltage (200 V) for 54 min and stained with ethidium bromide [16, 17].
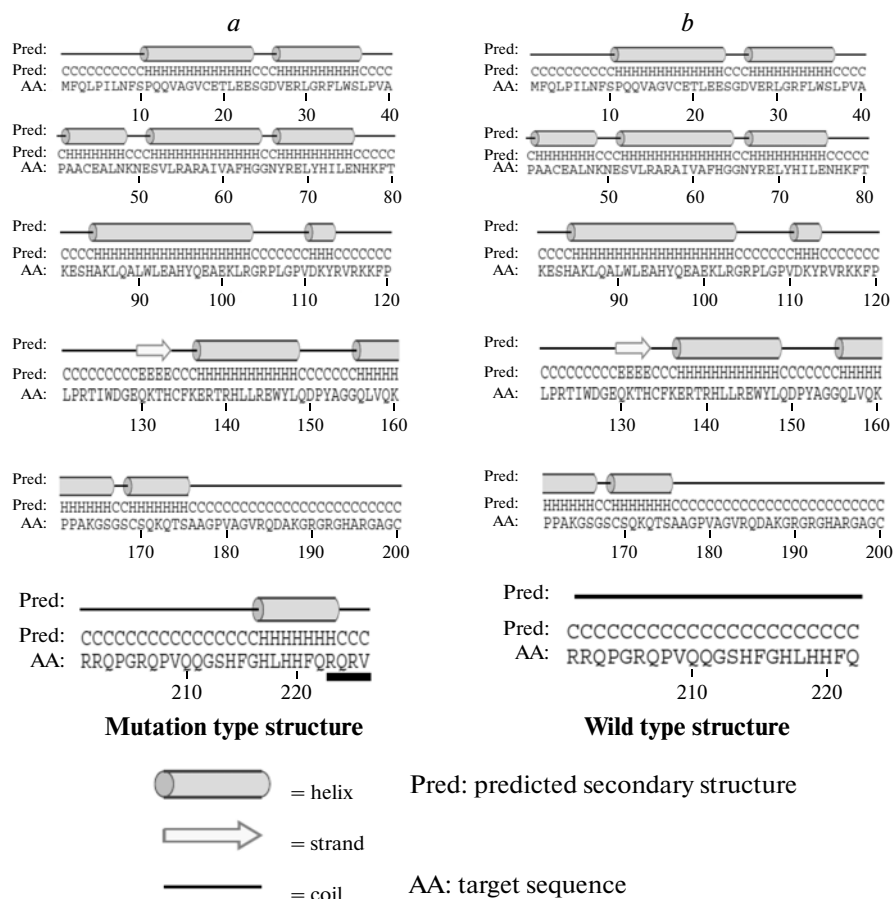
Fig. 1. Predicted patterns of the secondary structure of the bovine SIX6 homeobox protein by PSIPRED online software. *a* − Secondary structure of the mutant type; *b* − secondary structure of the wild type. Note: the underlined part represents the extended amino acid sequence.

Bioinformatics analysis of bovine SIX6 homeoprotein. This mutation leads to an extension of the encoded protein by four amino acids. The secondary structure of both types of the bovine SIX6 homeoprotein (wild type and mutant type) was preliminary predicted and compared by the online prediction software: the PSIPRED Server (http://bioinf4.cs.ucl.ac.uk:3000/psipred/) and APSSP2 Server (http://www.imtech.res.in/raghava/apssp2/) (Fig. 1) [18−20]. Alignment of the amino acid sequences of the SIX6 homeoprotein from humans, mice, sheep and cattle were analyzed by BioXM software (version 2.6) (http://home.njau.edu.cn/~bioxm) (Fig. 2).

Statistical analysis. Genotypic and allelic frequencies at the *SIX6*-HhaI locus and the Hardy−Weinberg equilibriums were calculated. Population genetic indexes, namely, gene heterozygosity and homozygosity, effective allele numbers and PIC (Polymorphism Information Content) were calculated by Nei's method [21]; SPSS software (version 16.0, USA) was applied to analyze the relationships between this polymorphism and growth traits in the NY population. Statistical analysis was performed on the basis of records of body weight, body length, body height, heart girth, birth weight, hucklebone width and average

daily gain in cattle. An effect associated with farm, sex and season of birth (spring versus fall) were not consistent with the linear model, as the preliminary statistical analyses indicated these effects did not have a significant influence on the variability of the traits in these breeds. Therefore, the following model was used to analyze the association of different genotypes with the growth traits [22, 23]: $Y_{iklm} = \mu + S_i + A_k + G_l + (AG)_{kl} + E_{iklm}$, where Y_{iklm} was the trait measured on each of the iklmth animal, μ was the overall population mean, S_i was the fixed effect associated with the ith sire, A_k was the fixed effect due to the kth age, G_l was the fixed effect associated with the lth genotype, $(AG)_{kl}$ was the interaction between the kth age and the lth genotype and E_{iklm} was the random error.

RESULTS AND DISCUSSIONS

In this study we identified a novel mutation (NC_007308: g 2015T > C) in the C-terminal domain of proteins encoded by the bovine *SIX6* gene by virtue of DNA sequencing and HhaI-ACRS-PCR. Sequence comparison of the genotypes (TT, TCA and CC) in the *SIX6* gene is given in Fig. 3. The electrophoretic patterns
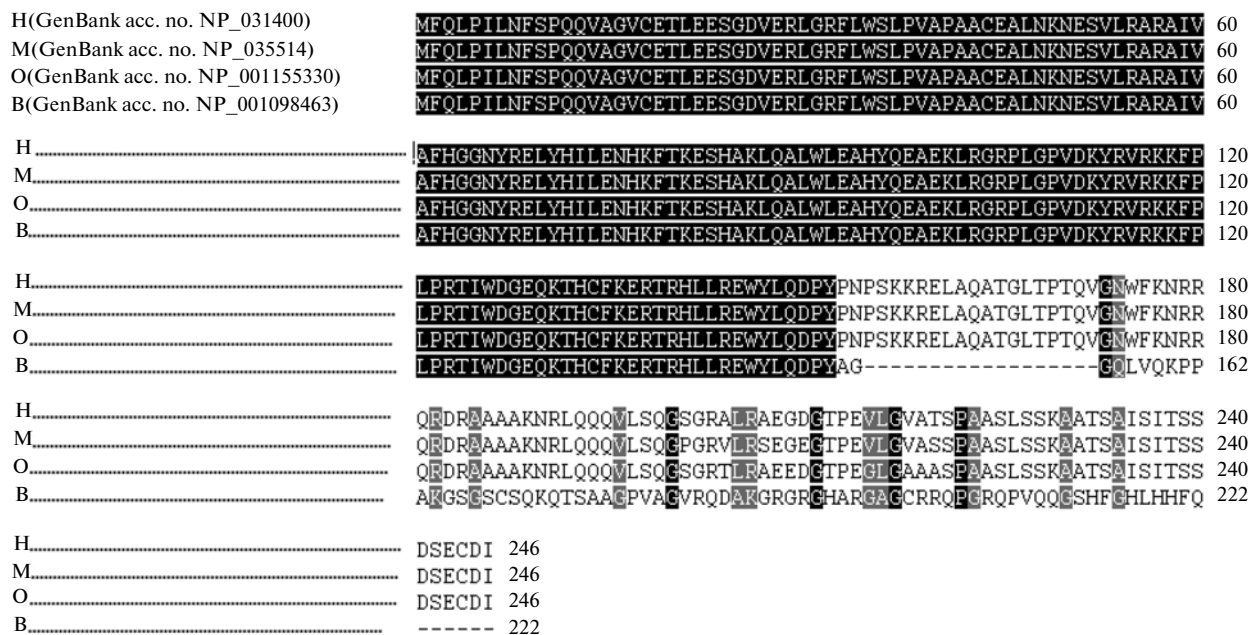
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H(GenBank acc. no. NP_031400)  MFQLPILNFSPQQVAGVCETLEESGDVERLGRFLWSLPVAPAACEALNKNESVLRARAIV  60
M(GenBank acc. no. NP_035514)  MFQLPILNFSPQQVAGVCETLEESGDVERLGRFLWSLPVAPAACEALNKNESVLRARAIV  60
O(GenBank acc. no. NP_001155330) MFQLPILNFSPQQVAGVCETLEESGDVERLGRFLWSLPVAPAACEALNKNESVLRARAIV  60
B(GenBank acc. no. NP_001098463) MFQLPILNFSPQQVAGVCETLEESGDVERLGRFLWSLPVAPAACEALNKNESVLRARAIV  60

H......................................AFHGGNYRELYHILENHKFTKESHAKLQALWLEAHYQEAEKLRGRPLGPVDKYRVRKKFP  120
M......................................AFHGGNYRELYHILENHKFTKESHAKLQALWLEAHYQEAEKLRGRPLGPVDKYRVRKKFP  120
O......................................AFHGGNYRELYHILENHKFTKESHAKLQALWLEAHYQEAEKLRGRPLGPVDKYRVRKKFP  120
B......................................AFHGGNYRELYHILENHKFTKESHAKLQALWLEAHYQEAEKLRGRPLGPVDKYRVRKKFP  120

H......................................LPRTIWDGEQKTHCFKERTRHLLREWYLQDPYPNPSKKRELAQATGLTPTQVGNWFKNRR  180
M......................................LPRTIWDGEQKTHCFKERTRHLLREWYLQDPYPNPSKKRELAQATGLTPTQVGNWFKNRR  180
O......................................LPRTIWDGEQKTHCFKERTRHLLREWYLQDPYPNPSKKRELAQATGLTPTQVGNWFKNRR  180
B......................................LPRTIWDGEQKTHCFKERTRHLLREWYLQDPYAG------------------GQLVQKPP  162

H......................................QRDRAAAAKNRLQQQVLSQGSGRALRAEGDGTPEVLGVATSPAASLSSKAATSAISITSS  240
M......................................QRDRAAAAKNRLQQQVLSQGPGRVLRSEGEGTPEVLGVASSPAASLSSKAATSAISITSS  240
O......................................QRDRAAAAKNRLQQQVLSQGSGRTLRAEEDGTPEGLGAAASPAASLSSKAATSAISITSS  240
B......................................AKGSGSCSQKQTSAACPVAGVRQDAKGRGRGHARGAGCRRQPGRQPVQQCSHFGHLHHFQ  222

H......................................DSECDI  246
M......................................DSECDI  246
O......................................DSECDI  246
B......................................------  222
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Fig. 2. Multiple alignment of amino acid sequences of the human, mouse, sheep and bovine proteins by BioXM2.6 software. Note: H − *Homo sapiens*; M − *Mus musculus*; O − *Ovis aries*. Black represents the same amino acid sequences; Gray represents similar amino acid sequences.
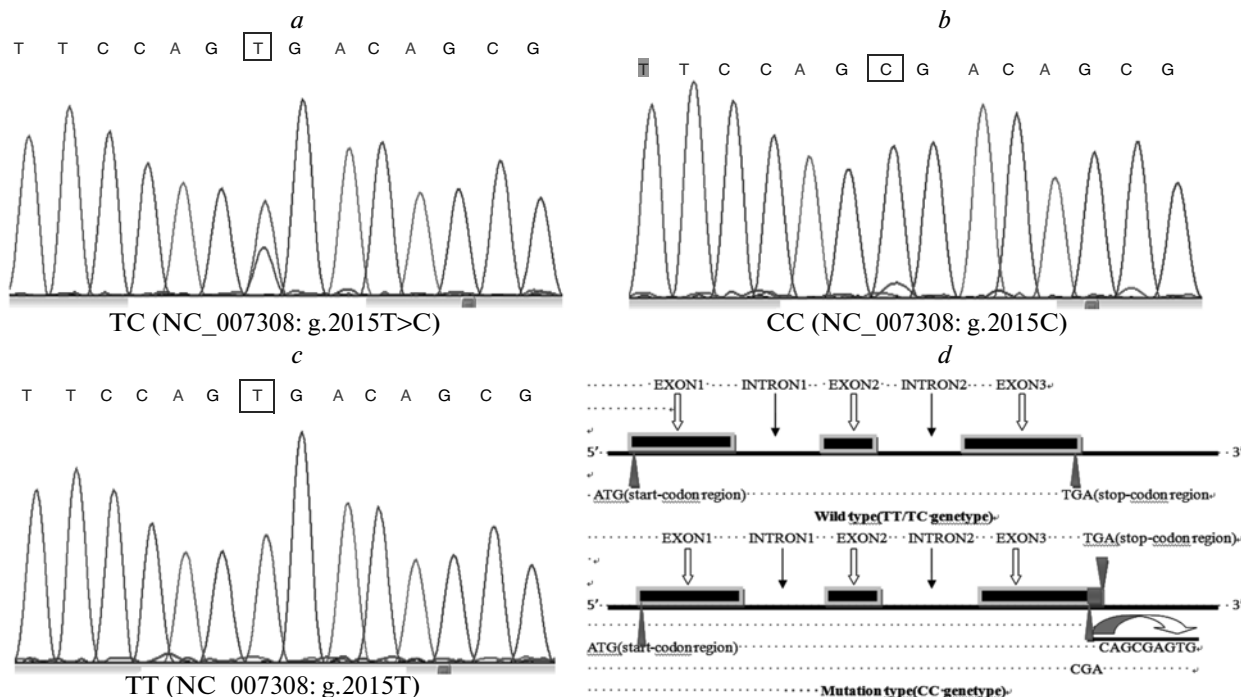
a

T T C C A G [T] G A C A G C G

TC (NC_007308: g.2015T>C)

b

T T C C A G [C] G A C A G C G

CC (NC_007308: g.2015C)

c

T T C C A G [T] G A C A G C G

TT (NC_007308: g.2015T)

d

EXON1····· INTRON1··· EXON2··· INTRON2··· EXON3
ATG(start-codon region)   TGA(stop-codon region)
Wild type(TT/TC genotype)

EXON1···· INTRON1··· EXON2··· INTRON2··· EXON3 ··· TGA(stop-codon region)
ATG(start-codon region)   CAGCGAGTG
CGA
Mutation type(CC genotype)

Fig. 3. Sequence of the mutation (T > C) site, sequence comparison of the three genotypes in *SIX6* gene and the electrophoresis patterns. *a* − Sequencing result of the TC genotype (NC_007308:g.2015T > C); *b* − sequencing result of the CC genotype (NC_007308:g.2015C); *c* − sequencing result of the TT genotype (NC_007308:g.2015T); *d* − patterns of the ORF shift and extension by twelve bases between the wild type and mutant type within the bovine *SIX6* gene.
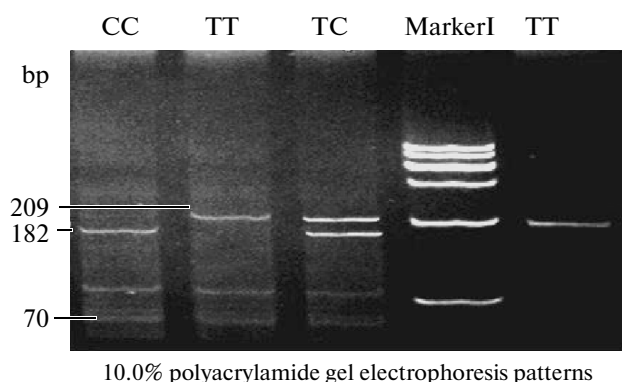
10.0% polyacrylamide gel electrophoresis patterns

Fig. 4. 10.0% polyacrylamide gel (PAGE) electrophoresis patterns of the HhaI ACRS-PCR analysis of the stop-codon locus within the bovine *SIX6* gene; CC genotype (182, 70 and 27 bp); TT genotype (209, 70 bp); TC genotype (209, 182, 70 and 27 bp); Marker I (100, 200, 300, 400, 500, 600 bp); TT genotype (209, 70 bp). Note: CC and TC showed in[M2]visible fragments 27 bp.

of HhaI-ACRS-PCR analysis within the *bovine SIX6* gene is shown in Fig. 4, the 278 bp PCR products including the stop-codon locus were amplified with P6 primer pairs and digested by HhaI endonuclease. However, there was a normal HhaI restriction site (NC_007308: g 1847) besides the one created in the PCR products. The novel mutation could lead to an ORF shift and extension of the encoded protein by four amino acids (Arg^{223}-Gln^{224}-Arg^{225}-Val^{226}) in the 3' untranslated region of the bovine *SIX6* mRNA (Fig. 3*d*). Other mutations of stop-codon which play an important role in the development of organs and tissues have been reported and studied in human and other animals [24, 25]. Therefore, the novel mutation might have an influence on the development of the bovine pituitary gland and some basic physiological indexes.

The frequencies of allele "C" varied from 0.255 CH to 0.614 (HA) in different cattle breeds, this reflected that the allelic frequencies of the bovine *SIX6* gene are tightly

associated with different bovine performance traits. CH and QC cattle has a long selection history for milk and beef respectively, but HA is just a foreign breed improved in China. Analysis of genetic indexes indicated that the genetic diversity in the analyzed populations (Table 2) was not very high (PIC < 0.5).

Secondary structure predictions have shown a new helix in the C-terminal domain of the proteins encoded by the mutant type. It has been reported that the *SIX6* genes are highly conserved and are involved in a wide variety of developmental processes and diseases. So, the CT extension may be important for providing structural stability to the third helix of the homeodomain as its presence increases the affinity of the HD for DNA [7, 26, 27]. The change in the spatial structure of the SIX6 homeobox protein probably has an effect on the development of the bovine pituitary gland. Meanwhile, alignment of the mRNA sequences from cattle, humans, mice and sheep in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) database has also shown that they all have some sequence variation in exon 3 and the flanking region. Multiple amino acid sequence alignment of human, mouse, sheep and bovine SIX6 homeobox protein showed the lack of 24 amino acids. All of the compared sequences had the same NT and SD, but the HD and CT were different. So, the function of the mutated SIX6 homeoprotein and protein–protein interactions remained stable, but the ability for DNA binding and other traits had changed with the variation of HD and CT in these Chinese cattle.

Subsequently, the relationships between this novel polymorphism of the bovine *SIX6* gene and productive traits (birth weight, body weight, body height, body length, heart girth, hucklebone width and average daily gain) was studied on the NY breed at 6-, 12-, 18-, and 24-months of age (Table 3). No statistically significant differences were observed in the analyzed population ($P > 0.05$) (data not shown). Although the mutation in the stop-codon affected the spatial structure of the SIX6 homeobox protein, DNA binding ability and other traits of

Table 2. Genotypic and allelic frequencies and genetic indexes of the polymorphism site in five cattle populations

Breeds Loci (2105 nt)	Genotype allele	TT	TC	CC	T	C	Ho	He	Ne	PIC	χ^2
QC ($n = 236$)	Numbers	128	92	16			0.613	0.387	1.632	0.312	0.009
	Frequencies	0.542	0.390	0.068	0.737	0.263					
JX ($n = 435$)	Numbers	217	166	52			0.572	0.428	1.748	0.336	5.120
	Frequencies	0.500	0.382	0.120	0.690	0.310					
NY ($n = 269$)	Numbers	140	117	12			0.613	0.387	1.631	0.312	4.169
	Frequencies	0.520	0.435	0.045	0.738	0.262					
CH ($n = 94$)	Numbers	48	44	2			0.620	0.380	1.614	0.308	2.308
	Frequencies	0.511	0.467	0.022	0.745	0.255					
Hasake ($n = 44$)	Numbers	2	30	12			0.526	0.474	1.902	0.362	8.438
	Frequencies	0.045	0.682	0.273	0.386	0.614					

Note: He — heterozygosis; Ho — Homozygosis; Ne — effective allele numbers; PIC — polymorphism information content.

Table 3. Association of the polymorphism at the mutation locus of *SIX6* gene with growth traits in NY cattle (Mean ± Standard Deviation)

Trait, kg	TT(140) (Mean ± SD)	TC(117) (Mean ± SD)	CC(12) (Mean ± SD)	*P* value
Birth weight	30.49 ± 2.82	29.72 ± 2.32	28.17 ± 0.76	>0.05
Body weight at 6 months	162.77 ± 16.38	156.12 ± 21.84	160.67 ± 15.37	>0.05
Average daily gain at 6 months	0.73 ± 0.09	0.70 ± 0.12	0.73 ± 0.09	>0.05
Body weight at 12 months	225.21 ± 24.47	221.12 ± 20.32	232.67 ± 28.57	>0.05
Average daily gain at 12 months	0.34 ± 0.10	0.36 ± 0.13	0.40 ± 0.19	>0.05
Body weight at 18 months	303.32 ± 30.64	292.61 ± 27.77	296.33 ± 21.55	>0.05
Average daily gain at 18 months	0.43 ± 0.25	0.39 ± 0.19	0.35 ± 0.24	>0.05
Body weight at 24 months	371.30 ± 45.13	368.44 ± 32.30	321.00 ± 32.51	>0.05
Average daily gain at 24 months	0.37 ± 0.30	0.42 ± 0.25	0.13 ± 0.13	>0.05

the transcription regulation mechanism, the main impact probably took place in the bovine embryo period and postnatal brain development and is thus associated with other biophysical and biochemical indexes.

To summarize, we have reported and confirmed a novel mutation in the TGA stop-codon of the bovine *SIX6* gene in 1078 healthy individuals from five breeds. Analysis of their secondary structure has shown that there is a new helix in the C-terminal domain of the proteins encoded by the mutant type; further studies on the specific function of the *SIX6* gene in cattle should be carried out. Therefore, this novel mutation of the bovine *SIX6* gene should be used for deeper functional analysis in the future.

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