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Article

A Missense Mutation in *KCNJ12* Was Strongly Associated with Beef Cattle Stature

Jie Cheng, Wenwen Peng, XiuKai Cao, Yongzhen Huang, Xianyong Lan, Chuzhao Lei, Hong Chen *

College of Animal Science and Technology, Northwest A&F University, Yangling, Shaanxi 712100, China; chengjie9412@163.com(J.C.); 18700481218@163.com(W.P.); cxkai0909@163.com(X.C.); hyzsci@126.com(Y.H.); lan342@126.com(X.L.); leichuzhao1118 @126.com (C.L.)

* Correspondence: chenhong1212@263.net; Tel.: +86-0298-709-2102

Simple Summary: A central goal of livestock genomic study is to find causal genes underlying economic traits and identify effective variations which can be used as molecular markers for livestock breeding. Cattle *KCNJ12* gene is an important candidate gene. To date, however, there is no report about its missense mutation maker in cattle stature. In this study, missense mutation in cattle *KCNJ12* was firstly verified, which leads to the change of its protein sequence. Further, significant association was detected between the mutation of *KCNJ12* and cattle stature and the mutation in *KCNJ12* can be used as molecular marker in beef breeding program. Besides, the expression analysis of *KCNJ12* gene has revealed high abundance in muscle and potential roles in bovine myocytes differentiation which can be as a foreshadowing for future research on the mechanism.

Abstract: Potassium inwardly-rectifying channel, subfamily J, member 12 *(KCNJ12)* gene is one promising candidate for economic traits because of its crucial roles in myoblast development. Here, a missense mutation (Cys>Arg), was firstly detected to locate in exon 3 of *KCNJ12* from three Chinese cattle breeds by DNA-pool sequencing. Then, we performed the association analysis of this SNP with stature in three Chinese cattle populations (n = 820). Significantly positive correlation was revealed by reduced animal general linear model and the genotype of CC is the most excellent genotype in three breeds. Further, we measured the expression profiling of the *KCNJ12* gene in various cattle tissues and primary bovine skeletal muscle cells. Ubiquitous expression with high abundance in muscle was observed. Further, in primary bovine skeletal muscle cells, the *KCNJ12* mRNA expression was gradually up-regulated in differentiation medium (DM) compared with that in growth medium (GM), suggesting that *KCNJ12* gene is involved in bovine myocyte differentiation. Conclusively, *KCNJ12* gene is a functional candidate gene which can be used as molecular marker for beef cattle breeding.

Keywords: *KCNJ12*; SNP; myoblast differentiation; stature; Chinese beef cattle

1. Introduction

The understanding about growth and development of skeletal muscle is one of the most important goals in animal and meat science. Meat characteristics are directly affected by many factors among which genetic factors are of prime importance because genetic improvement is permanent and cumulative when inherited by the next generations. Genetic variation in the livestock is known to be a resource of utmost importance. Therefore, identifying the causal loci of meat productivity and quality is a subject of intense research, and to date only a fraction of these loci have been discovered [1]. Therefore, it is urgent to discover these loci and improve their understanding of the molecular mechanisms in skeletal muscle development.

Potassium inwardly-rectifying channel, subfamily J, member 12 (*KCNJ12*), belongs to the inward-rectifier potassium channel family which includes the strong inward-rectifier channels (Kir2.x), the G-protein-activated inward-rectifier channels (Kir3.x) and the ATP-sensitive channels (Kir6.x). The *KCNJ12* gene encodes an inwardly rectifying K+ channel protein, Kir2.2, and can combine with sulphonylurea receptors. Inwardly rectifying K+ channel may be blocked by divalent cations and is one of the multiple inwardly rectifying channels contributing to the cardiac and nerve inward rectifier current (IK1) [2]. Inward rectifying potassium channel, activated by phosphatidylinositol 4,5-bisphosphate, probably participates in controlling the resting membrane potential in electrically excitable cells and establishing action potential waveform and excitability of neuronal and muscle tissues.

Motoneurons are important for regulating the function and properties of skeletal muscle. Potassium inward rectifier (Kir) channels are important to establish the resting membrane potential and regulating the muscle excitability. Thus the *KCNJ12* gene possibly involved in the regulation of muscle membrane properties and excitation-contraction coupling [3]. Expectedly, mutations in Kir channels can cause disorders affecting the heart and skeletal muscle, such as arrhythmia and periodic paralysis in human [4]. For example, non-synonymous coding single nucleotide polymorphisms (SNPs) of *KCNJ12* are associated with pathogenesis of Rhabdomyosarcomas (RSCs) [5]. Besides, the expression level of *KCNJ12* is relevant to Dilated cardiomyopathy (DCM), and the number of Kir2.2 channels were decreased in DCM ventricles [6]. What's more, diseases associated with *KCNJ12* also include Smith-Magenis Syndrome and so on [7].

Based on the GWAS of copy number variations (CNVs) and growth traits in Bos indicus, Zhou (2016) has reported that *KCNJ12* could be a candidate gene for muscling through the modulation of muscle contraction and food intake. [8]. Additionally, the two CNVs of *KCNJ12* were significantly associated with stature in four Chinese cattle populations, including NY (Nanyang cattle), JX (Jiaxian cattle), JA (Jian cattle) and GF (Guangfeng cattle) [9]. Although *KCNJ12* gene located at *Bos taurus* autosome 19 (BTA19): 35,955,796-35,991,035 bp (AC_000176) has been widely proved to be an important candidate for cattle stature, no reports on the SNP markers of *KCNJ12* gene have been investigated in previous studies. Therefore, the aim of this study was to: 1) analyze the genetic polymorphisms of SNPs in *KCNJ12* by using DNA-pool sequencing and forced PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) in three Chinese cattle breeds; 2) establish the significant association between the mutation of *KCNJ12* gene and cattle stature; 3) examine the relative expression of *KCNJ12* gene in different tissues and time-course of myoblast differentiation by RT-qPCR (reverse transcription-quantitative polymerase chain reaction). The results will provide new insights into the transcriptional regulation of the cattle *KCNJ12* gene and the potential applications in cattle breeding.

2. Materials and Methods

2.1. Ethics Statement

The protocols used in this study and animals were approved by the Administration of Affairs Concerning Experiment Animals (Ministry of Science and Technology, China, 2004) and Faculty Animal Policy and Welfare Committee of Northwest A&F University (FAPWC-NWAFU).

2.2. Animals and Data Collection

A total of 820 cows (*Bos taurus*) were used in this study, including Pinan cattle (PN, n = 372), Jin'nan cattle (JN, n = 205) and Xia'nan cattle (XN, n = 243). The JN cattle is the main local beef cattle breed in China. The PN and XN are well-known cultivate breeds, both of which have the features of superior growth and meat traits. The animals of each breed were selected to be unrelated for at least three generations to exclude sire effects. After weaning at 6 months of age, these animals were fed *ad libitum* under comfortable conditions which were half-grazing and half-house feeding without straw-covered. Records of cattle stature included: 1) withers height (WH), body oblique length (BOL), hip width (HW), chest girth (CG), thurl width (TW) and rump length (RL) in PN cattle; 2)

withers height (WH), body oblique length (BOL), hip width (HW), paunch girth (PG), chest girth (CG), cannon bone circumference (CBC) and body weight (BW) in XN cattle; 3)withers height (WH), body oblique length (BOL), hip width (HW), chest girth (CG) and rump length (RL) in JN cattle [10]. PN and JN groups were cows with different ages, and XN group included adult cattle with both genders.

2.3. DNA isolation and genomic DNA sequencing

Genomic DNA were extracted from 820 heparin-treated whole blood samples and 30 muscle tissues according to standard procedures [11]. The genomic DNA was diluted to 50 ng/μL which was measured by spectrophotometer ($1.6 < OD_{260/230} < 2.0$ and $2.0 < OD_{260/280} < 3.0$) and then stored at -80 °C [11]. Fifty DNA samples of each breed were randomly selected to construct three DNA pools, respectively. Based on the reference sequence in the NCBI database (GenBank accession no. AC_000176), eleven primers (P1-P11) was designed to screen variations in exon (Table S1) for PCR amplification from cattle genomic DNA, and PCR products were detected by 2% agarose gel electrophoresis. For example, the bands amplified by P3 primer was clearly showed in Figure S1 using marker D2000 in which the length of PCR product is 1177 bp. Then the PCR products were sent to the sequencing company to complete the subsequent sequencing work in both directions (Shenggong, Shanghai, China).

2.4. Genotyping of four variations within cattle KCNJ12 gene

After screening the mutation, one pair of primers (Table 1) were redesigned to genotype the novel SNP (g.35989944T>C) for each individual. Given that the SNP locus has no natural restriction enzyme cutting sites, we use forced PCR-RFLP method to genotype the SNP. In detail, *Pst I* site (CTGCA ↓ G) was created by changing the "AG" to "CT" in reward primer. After introducing the mismatch, the SNP could be genotyped by PCR products digested by *Pst I* [12]. The digested were detected by electrophoresis of 3.5 % agarose gel stained with Nucleic Acid Dyestuff and using Marker Ⅰ which includes 100, 200, 300, 400, 500 and 600bp bands.

Table 1. PCR primer sequences and approach for identification of SNP in *KCNJ12*.

Name	Chr Position	Primers Sequence (5′–3′)	Genotyping Methods	Tm (˚C)	Restriction enzyme	Genotype pattern (bp)
SNP	g.35989944 T>C	F:CGAGGAGTGCCCGGTGGCGGTGTTCAT R:TAGGTTGCCCACGCGCCACATGC***T***GC	PCR-RFLP	57	*Pst I*, CTGCA ↓ G	199 (176 + 23)

The italic and bold base means the introduction of mismatch.

2.5. Statistical analysis

Population parameters such as genotypic frequencies, allele frequencies, homozygosity (Ho), heterozygosity (He), effective allele numbers (Ne) and polymorphism information content (PIC) were calculated by online software (http://www.msrcall.com/Gdicall.aspx). The $\chi 2$ tests for Hardy-Weinberg equilibrium (HWE) were calculated by PopGene software [13].

The association analysis was performed using the full animal general linear model (GLM) followed by the reduced animal model which was used in the final analysis. The full animal model included fixed effect of genotype, sex, farm, and random effects of age. Association analysis between genotypes and cattle stature was performed by SPSS 18.0 (Statistical Product and Service Solutions, Version 18. 0 Edition) using the following established reduced model after exclusion of non-significant confounders which was as follows:

$$\mathbf{Y_{ijlm} = u + A_i + G_j + S_l + e_{ijlm}} \quad (1)$$

where Y_{ijlm} is the observation of the cattle stature, A_i is the random effect of age, G_j is the fixed effect of genotype, S_l is the fixed effect of sex and e is the random residual error. For PN cattle and JN cattle, S=0, and For XN cattle, A=0. Notably, sire effect was not included in the model considering

that these animals were unrelated for at least three generations. Multiple tests were not corrected at $P = 0.05$ and $P = 0.01$.

2.6. Cell culture and induction differentiation

Primary bovine myoblasts were isolated from bovine skeletal muscle of limbs in 90-day fetal cattle by the method of enzyme digestion. To promote myoblasts proliferate, cells were reseeded in 12-well dishes and cultured in growth medium, which included high-glucose DMEM supplemented with 20% fetal bovine serum and double antibiotics (1% penicillin and streptomycin) at 37°C under a 5% CO2 atmosphere.

To induce myoblasts differentiation, when cells were at ~95% density marked as differentiation 0 day, the growth medium was replaced with 2% horse serum medium with 1% penicillin/streptomycin. The cells were refreshed with new medium every 24 hours, and were cultured for -1, 0, 1, 2, 4 days to induce differentiation prior to RNA extraction.

2.7. RNA extraction, cDNA synthesis and expression analyses

Total RNA was extracted from six tissues including heart, liver, spleen, lung, kidney and muscle which were collected from three 90-day cattle and differentiating myoblasts using Trizol Reagent (TaKaRa, Japan) following the manufacturer's protocol [14]. RNA integrity was assessed by electrophoresis on 1.0% agarose gel, and RNA purity was verified by measuring the absorbance at 260 and 280 nm by ND-1000 spectrophotometer (NanoDrop Technologies, USA). The cDNA for each sample was synthesized from an equal amount of total RNA (500 ng) by PrimeScript RT reagent kit (TaKaRa, Japan) following the manufacturer's protocol. To reveal the correlations between genotypes and expression level, 30 muscle samples were also used to extract RNA and then revert to cDNA.

The mRNA expression levels of *KCNJ12*, *MYOD*, *MYOG*, *MYHC* and *GAPDH* were evaluated with SYBR® Premix Ex Taq™ kit (TaKaRa, Japan) by qPCR in Bio-Rad CFX96 RT-PCR System (Bio-Rad, Hercules, CA, USA). The amplification efficiencies of all primers were measured with serial dilutions of cDNA (0.005, 0.05, 0.5, 5, 50, and 500 ng), and PCR efficiencies are close for *KCNJ12*, *MYOD*, *MYOG*, *MYHC* and *GAPDH* gene-specific primers (Table 2). The *GAPDH* gene was chosen as the internal reference gene for the qPCR analysis. The expression levels were calculated by using $2^{-\Delta\Delta Ct}$. All experiments were repeated three times.

Table 2. PCR primer sequences of *KCNJ12* and *GAPDH* gene in cattle for qPCR.

Gene name	Primer sequences (5′–3′)
MyoD	F: ACGGCATGATGGACTACAGC R: AGGCAGTCGAGGCTCGACA
MyoG	F: CAAATCCACTCCCTGAAA R: GCATAGGAAGAGATGAACA
MyHC	F: TGCTCATCTCACCAAGTTCC R: CACTCTTCACTCTCATGGACC
KCNJ12	F: TGGGCAACCTACGCAAGAGC R: GCAGGATGGTGATGGGAGACA
GAPDH	F: CGACTTCAACAGCGACACTCAC R: CCCTGTTGCTGTAGCCAAATTC

3. Results

3.1. Identification of genetic variation in cattle KCNJ12 gene

By DNA-pooling PCR sequencing, only one missense mutation in *KCNJ12*, named as g.35989944T>C, was identified for the first time in three beef cattle breeds (Figure 1). The g.35989944T>C was located in exon 3 (Cys>Arg) and had three genotypes based on the PCR products

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digested by the *PstI*. As shown in Figure 2, the genotypes were classified as CC (199 bp), TC (199 bp and 176 bp) and TT (176 bp) according to the agrose gel electrophoresis analysis.

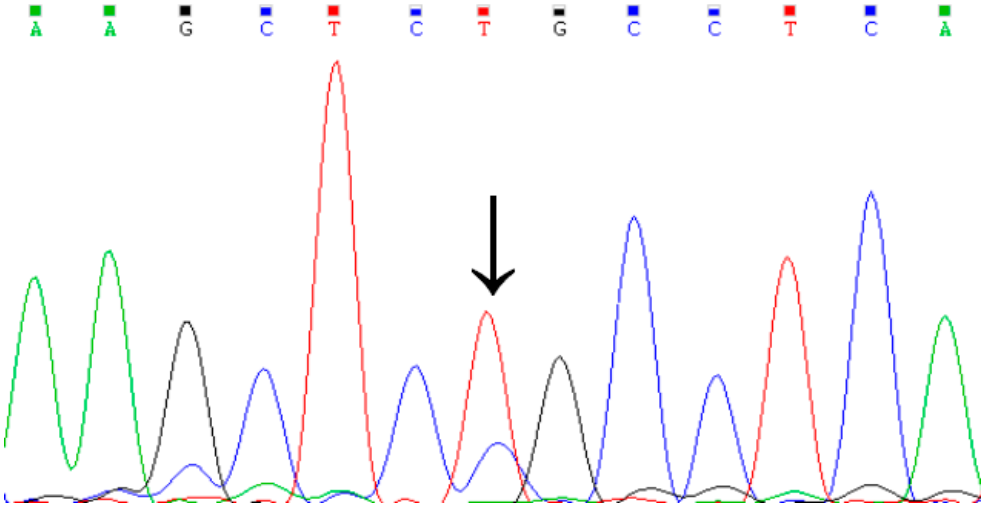

Figure 1. Sequencing result of g.35989944T>C in *KCNJ12* gene.

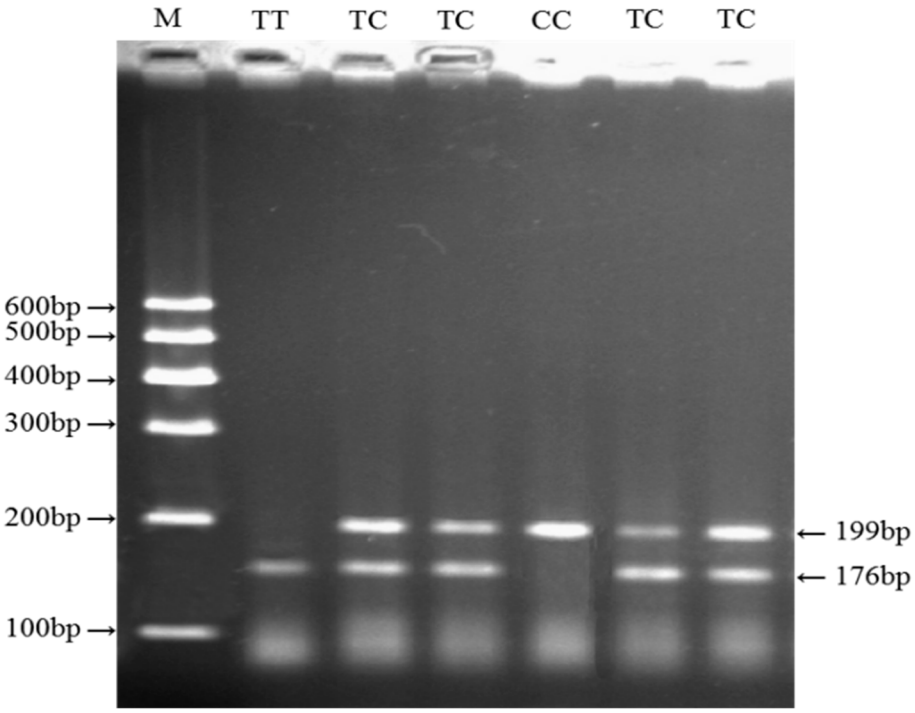

Figure 2. PCR-RFLP of g.35989944T>C by agrose gel eletrophresis.

3.2. Genotypes, allele frequencies and genetic diversity of the SNP in KCNJ12 gene

The sample sizes, genotypic frequencies, allelic frequencies, homozygosity (Ho), heterozygosity (He), effective allele numbers (Ne), and polymorphism information content (PIC) of the SNP in *KCNJ12* gene in the three cattle breeds were shown in Table 3. The results obtained from the preliminary analysis suggested that the frequencies of genotypes and alleles were different in three cattle breeds. The results also indicated that the SNP was polymorphic and were in medium genetic diversity in all these cattle breeds ($0.25 < PIC < 0.5$). The $\chi 2$ test indicated that the SNP (g.35989944T>C) in JN cattle, a local breed, was in HWE ($P > 0.05$) instead of XN and PN, two cultivated breeds. A locus keeping in HWE suggested allelic balance during long evolution and breeding.

Table 3. Population genetic indices of the SNP mutation.

name	Breeds (sizes)	Genotype frequencies			Allele frequencies		HWE-χ^2	*P*-value	Ho	He	Ne	PIC
		TT	TC	CC	T	C						
SNP	XN/243	0.082	0.506	0.412	0.335	0.665	4.455	*P*<0.05	0.554	0.446	1.804	0.346
g.35989944	JN/205	0.063	0.293	0.644	0.210	0.790	2.813	*P*>0.05	0.668	0.332	1.496	0.277
T>C	PN/372	0.167	0.602	0.231	0.468	0.532	16.301	*P*<0.05	0.502	0.498	1.992	0.374

PIC < 0.25, low polymorphism; 0.25 < PIC < 0.5, intermediate polymorphism; and PIC > 0.5, high polymorphism.

3.3. Association study of g.35989944T>C with cattle stature

The results of association analysis between the g.35989944T>C locus and cattle stature were visualized in Table 4 (JN cattle), Table 5 (XN cattle) and Table 6 (PN cattle). For JN cattle, the SNP was found to be significantly associated with the CG (P < 0.05), WH and HW (P < 0.01). Notably, WH was higher for individuals with genotype CC (129.61 ± 0.55 cm) than TC (127.00 ± 0.73 cm) and TT (124.36 ± 1.69 cm). These is no significant associations between CC and TT, which may be result from deviation. For XN cattle, the SNP was found to be extremely significantly associated with CG, PG and BW (P < 0.01). BW was exceedingly higher for individuals with genotype CC (471.91±10.42 cm) and TC (455.55 ± 7.24 cm) than TT (385.45 ± 12.52 cm). For PN cattle, the SNP was found to be markedly associated with the WH (P < 0.05), BOL, HW, CG, TW and RL (P < 0.01). WH was higher for individuals with genotype CC (126.70 ± 0.75 cm) and TC (124.59 ± 0.41 cm) than TT (122.59 ± 0.70 cm).

Table 4. Association between the *KCNJ12* variation and cattle stature in JN cattle.

locus	Genotypes	Body trait (Mean ± SE)				
		withers height	hip width	body oblique length	Chest girth	Rump length
g.35989944 T>C	CC	129.61±0.55^{A}	132.02±0.65^{A}	153.26±0.95	186.74±1.13^{a}	48.63±0.34
	TC	127.00±0.73^{B}	129.51±0.92^{B}	151.41±1.51	181.82±2.25^{b}	47.34±0.80
	TT	124.36±1.69^{A}	125.36±2.02^{A}	146.09±3.16	176.64±4.36^{a}	46.18±1.43
	P value	0.001	0.002	0.074	0.013	0.061

Different letters in the same row mean differ significantly (a, b: $P < 0.05$; A,B:$P < 0.01$), LSE is least square means, and SE is standard error.

Table 5. Association between the *KCNJ12* variation and cattle stature in XN cattle.

locus	Genotypes	Body trait (Mean ± SE)						
		withers height	hip width	body oblique length	Chest girth	paunch girth	cannon bone circumference	Body weight
g.35989944 T>C	CC							
	TC	129.26±0.92	135.58±0.83	148.52±4.61	185.16±2.42^{A}	217.60±1.54^{A}	18.51±0.18	455.55±7.24^{A}
	TT	127.00±1.02	135.63±1.60	148.45±0.22	176.55±2.54^{B}	205.64±3.22^{B}	18.00±0.39	385.45±12.52^{B}
	P value	0.383	0.246	0.751	0.003	0.001	0.467	<0.001

Different letters in the same row mean differ significantly (a, b: $P < 0.05$; A,B:$P < 0.01$), LSE is least square means, and SE is standard error.

Table 6. Association between the *KCNJ12* variation and cattle stature in PN cattle.

locus	Genotypes	Body trait (Mean ± SE)					
		withers height	body oblique length	hip width	Chest girth	thurl width	Rump length
g.35989944 T>C	CC	126.70±0.75^{a}	151.41±1.24^{A}	133.37±0.74^{A}	178.39±1.38^{A}	47.05±0.38^{A}	49.35±0.40^{A}
	TC	124.59±0.41^{a}	147.42±0.72^{B}	131.41±0.40^{B}	172.91±0.88^{B}	45.43±0.28^{B}	48.28±0.26^{B}
	TT	122.59±0.70^{b}	143.98±1.32^{C}	129.46±0.69^{C}	169.22±1.72^{B}	44.57±0.52^{B}	47.31±0.49^{B}
	P value	0.04	<0.001	0.001	<0.001	0.001	0.008

Different letters in the same row mean differ significantly (a, b: $P < 0.05$; A,B:$P < 0.01$), LSE is least square means, and SE is standard error.

3.4. Expression analyses of *KCNJ12* in cattle tissues

In order to study if the variation has influence on mRNA expression level, we firstly studied *KCNJ12* expression profiling in six tissues of 90-day cattle. After that, we performed the associations between *KCNJ12* genotypes and expression level in muscle.

The expression levels of *KCNJ12* in different tissues were detected using RT-qPCR. As Figure 3 showed, the mRNA abundance of cattle *KCNJ12* gene varied in different tissues with the highest expression level in muscle. Because the study concerned on the growth and development of muscle, then 30 muscle samples were used to test the potential correlations between different genotypes and *KCNJ12* gene mRNA expression level (Figure 4). We found that the expression of heterozygous individuals with TC genotype are significantly higher than individuals with homozygous TT or CC genotypes. However, there is no significant difference between the two homozygotes, which indicates that the heterozygous individuals have certain advantages.

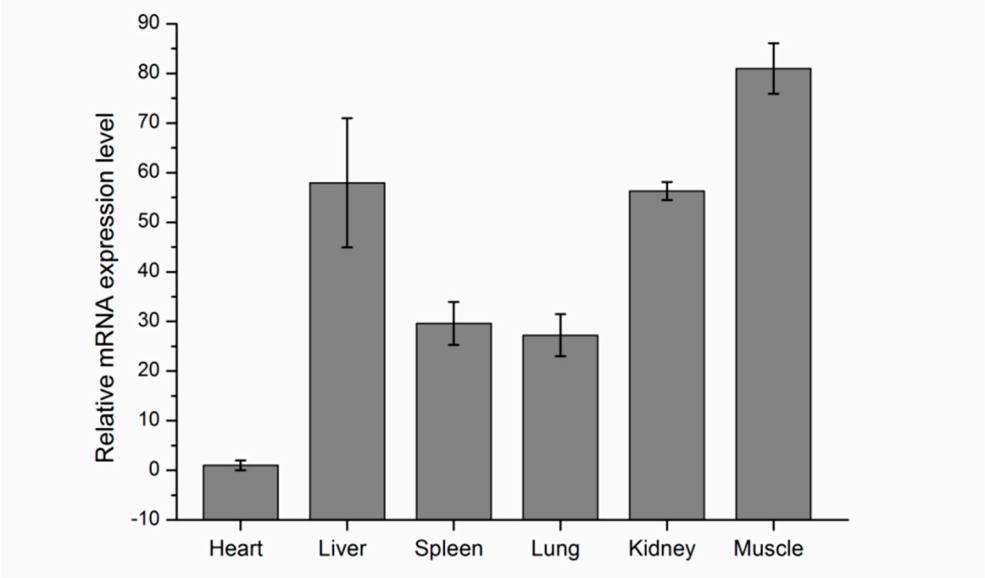

Figure 3. Expression profiling of *KCNJ12* in different tissues of 90-day cattle. The values are the averages of three samples calculated by $2^{-\Delta\Delta Ct}$. Error bars represent the standard error (SE) (n = 3) calculated by ΔCt.

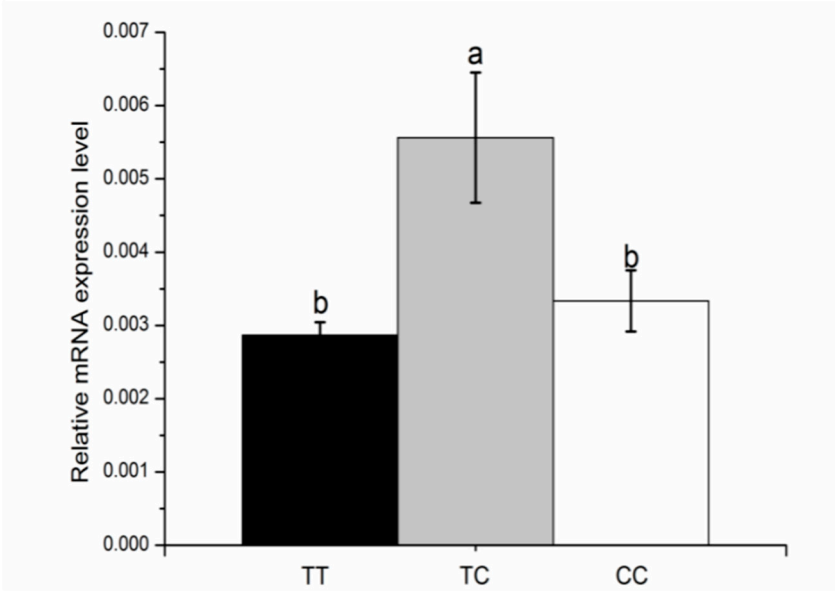

Figure 4. The effects of g.35989944T>C on *KCNJ12* gene expression in cattle muscle tissues.

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3.5. *KCNJ12* expression during primary bovine skeletal muscle cells differentiation

The expression levels of *KCNJ12*, *MYOD*, *MYOG* and *MYHC* in primary bovine skeletal muscle cells serve as an excellent model system to study muscle cell differentiation in vitro [15]. Relative expression levels of these genes were detected during differentiation of primary bovine skeletal muscle cells by RT-qPCR normalized to *GAPDH*. We found that the expression level of the four genes were different on different differentiation days (Figure 5). The expression of *KCNJ12* was gradually up-regulated in differentiation medium (DM) compared with that in growth medium (GM), with a slight decrease after differentiation day 1 (DM1), when myotubes were being formed. And this was similar to the expression of *MYOD*. Besides, the relative expression of *MYOD* at most was at DM1. In addition, the relative expression of *MYOG* was in a very high state for DM1, DM2 and DM3, and the peak value of *MYHC* appeared at the second day of differentiation (DM2).

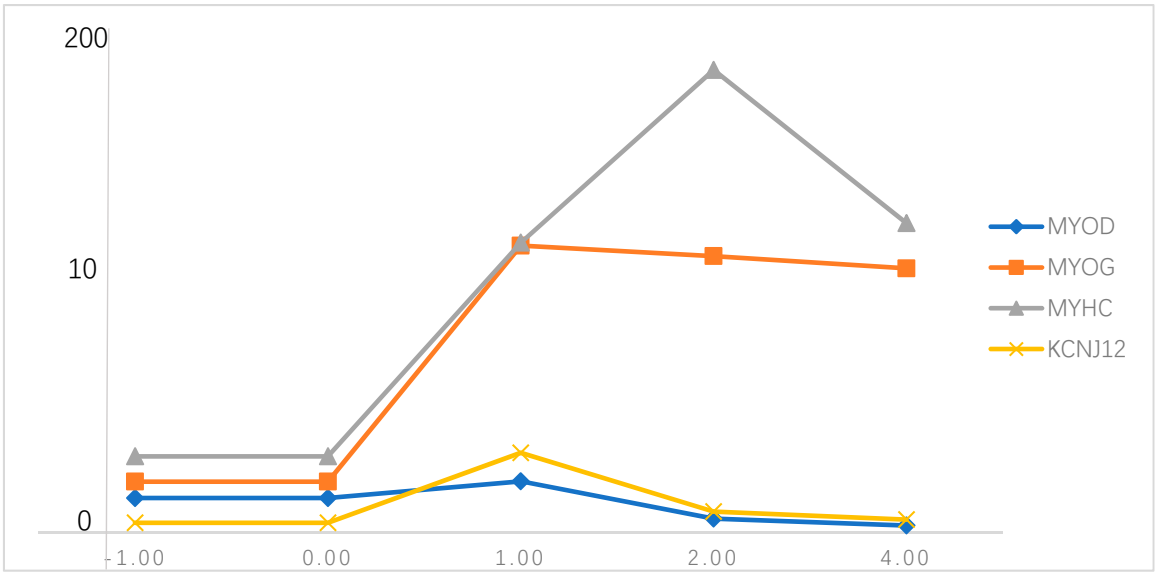

Figure 5. Expression of *KCNJ12*, *MYOD*, *MYOG* and *MYHC* gene during myoblast differentiation. Relative mRNA expression levels of *KCNJ12*, *MYOD*, *MYOG* and *MYHC* gene in different differentiation periods were analyzed by qPCR in primary bovine skeletal muscle cells. The mRNA expression levels of the four genes were normalized to *GAPDH*.

4. Discussion

We are in a phase of unprecedented progress in identifying genetic loci that cause variations in economic traits of livestock and more markers are required for the implementation of genomic selection in Chinese cattle [16]. In this study, we identified one SNP, a missense mutation of the bovine *KCNJ12* gene through DNA pool sequencing in three Chinese cattle breeds. The SNP in *KCNJ12* gene has abundant genetic diversity, which may be essential for production improvement. Recently, a considerable literature has grown up around the theme that missense mutations can influence the expression of gene [17] and the protein function [18,19]. The SNP in *KCNJ12* is a missense mutation located in exon 3 of the bovine *KCNJ12* gene, and may affect translation efficiency, thereby altering the function of the *KCNJ12* protein. Association analysis indicated that the SNP was significantly associated with cattle stature, in which the genotype of CC is the most excellent genotype in three breeds. This suggest a strategy for the breeding workers that individuals with CC genotype can be selected in offspring.

The mRNA level which occurs after transcription and before translation indicates the relationship between DNA and protein, so measurement of mRNA levels is typically performed to investigate the role of target genes [15]. Expression analysis of the bovine *KCNJ12* gene showed that *KCNJ12* was widely expressed in different tissues and particularly highly expressed in muscle (Figure 3), which implies that the *KCNJ12* gene may play a major role in cattle muscle development.

Besides, it can be seen from the data in Figure 4 that the relative expression of TC genotype was the highest in three genotypes, however CC genotype had better growth performance. One possible explanation for this might be that heterozygous individuals have certain advantages. For example, heterosis can promote the expression of *KCNJ12*, leading to that energy tend to K+ flowing and neurotransmitter transmission instead of myoblasts proliferation. Thus, phenotypes would develop in the opposite direction which was being decreased when the expression become increased.

Muscle progenitor cells differentiate into myoblasts, through proliferation, differentiation and fusion into multinucleated myotubes, eventually forming mature muscle fibers [20,21]. Muscle development is mainly regulated by a series of transcription factors which included paired box protein 3/7 (Pax3/7), myogenic regulator (MRFs) family (MYOD, myogenin, Myf5 and MRF4) [22,23] and myocyte enhancer factor 2 (MEF2) family (MEF2A, MEF2B, MEF2C and MEF2D) [20,24,25]. In addition, the muscle development is also regulated by other protein-coding genes directly or indirectly. Many published studies have reported that the *KCNJ12* (Potassium inwardly-rectifying channel), as the name suggests, is crucial in transmission of nerve impulses. As known, motoneurons are important for regulating the function and properties of skeletal muscle, so the gene is possible to be involved in the regulation of muscle development [26]. In our study, as we can see from Figure 5, the highest expression of *KCNJ12* was at DM1 which was similar to *MYOD*. The gene was up-regulated in differentiation medium (DM) compared with that in growth medium (GM), myotubes were being formed, which indicated that *KCNJ12* tends to induce differentiation of bovine skeletal muscle. Moreover, the highest expression of *MYOD* was at differentiation day 1 (DM1). The relative expression of *MYOG* was in a very high state for DM1, DM2 and DM3 and the peak value of *MYHC* appeared at the second day of differentiation (DM2). Therefore, we can deduce that cattle *KCNJ12*, *MYOD*, *MYOG* and *MYHC* were activated in different stages. This result is also consistent with a previous study that it's in pre-differentiation period and even post-proliferation that *MYOD* make efforts on muscle differentiation, and then *MYOG* and *MYHC* in C2C12 [27]. These data strongly suggested that *KCNJ12* polymorphisms could be used as molecular marker for marker-assisted selection in beef cattle breeding.

5. Conclusions

Association analysis was conducted between the mutation of *KCNJ12* and cattle stature in beef breeding program and CC genotype of g.35989944T>C is the most excellent genotype in all three breeds. The expression analysis of *KCNJ12* gene has revealed high abundance in muscle and potential roles in bovine myocytes differentiation. This study will provide some useful information for cattle breeding.

Supplementary Materials: Figure S1: Amplification of *KCNJ12* using P3 primers. Table S1. Primers information for PCR amplification of bovine *KCNJ12* gene.

Author Contributions: Conceptualization, J.C.; Data curation, W.P.; Formal analysis, J. C.; Investigation, X. C.; Methodology, X.C.; Project administration, H.C.; Resources, Y.H.; Software, X.L.; Supervision, X.L., Y.H. and C.L.; Validation, H.C.; Writing – original draft, J.C.; Writing – review & editing, X.C..

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Abbreviation List: WH, withers height; BW, body weight; BOL, body oblique length; HW, hip width; PG, paunch girth; CBC, cannon bone circumference; CG, chest girth; TW, thurl width; RL, rump length; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription PCR; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; QTL, quantitative trait loci; HWE, Hardy-Weinberg equilibrium; PN, Pinan cattle; XN, Xia'nan cattle; JN, Jinnan cattle; JX, Jiaxian cattle; NY, Nanyang cattle; GF, Guangfeng cattle; JA, Jian cattle.

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