



Research Article

Identification of genetic variants the CCKAR gene and based on body measurement and carcass quality characteristics in Qinchuan beef cattle (*Bos taurus*)



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ABSTRACT

Background: This study aimed to explore genetic polymorphisms of the CCKAR gene and their relationship with the growth and development of Qinchuan cattle which could be used as molecular markers for the improvement of the breeding of Qinchuan cattle.

Results: Here, we have identified seven single nucleotide polymorphisms (SNPs) at loci g. 1463 C>G; g. 1532 T>A; g. 1570 G>A; g. 1594 C>A; g. 1640 T>C; g. 1677 G>C; and g. 1735 C>T in the coding region of the bovine CCKAR gene. The frequencies identified on allelic and genotypic characteristics have shown that all seven SNPs diverged from the Hardy-Weinberg-Equilibrium. The SNP2, SNP3, SNP6 and SNP7 had the lowest polymorphism information content values, and remaining SNPs were found to be moderate (0.25 < PIC < 0.50). The genotype CG in SNP1 at loci g.1463 C>G had the greatest association with WH, HW, CD and CCF, while the genotype TA at the very same loci was associated with BFT, ULA and IMF content in Qinchuan cattle. The CCKAR gene expression level in adipose tissue, small intestine, liver and skeleton muscle was found to be higher, whereas, the expression level of mRNA in organs of other digestive system including reticulum, abomasum and omasum was moderate. Some expression of CCKAR mRNA was found in the large intestine, kidney and rumen.

Conclusions: In summary, our finding suggested that the CCKAR gene could be used as a potential candidate for the improvement of carcass quality and body measurements of Qinchuan cattle.

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Abbreviations: CCKAR, cholecystokinin A-receptor; SNPs, single nucleotide polymorphisms; HWE, Hardy-Weinberg-Equilibrium; WH, withers height; HW, hip weight; CD, chest depth; CCF, chest circumference; IMF, Intramuscular fat; CNS, central nervous system; CDS, coding sequence; LD, linkage disequilibrium; GLM, general linear model; BFT, back fat thickness; ULA, ultrasound loin area; PIC, polymorphism information content; NBCIC, National Beef Cattle Improvement Center.

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1. Introduction

In China, the Qinchuan is a popular breed for beef due to its high body weight, genetic stability and its environmental adaptability. However, compared to exotic cattle breeds, Qinchuan cattle have low economic benefits due to low IMF [1,2,3,4,5,6,7]. Generally, it has been considered that the percentage of IMF has a positive effect on the sensory quality such as the juiciness and the tenderness of the meat [8,9,10].

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Traditional breeding schemes for genetic gain have substantial lag periods before benefits are seen. Thus, advanced molecular technologies have been widely used in cattle breeding and genetic mapping to achieve effective and sustainable genetic gains for economically important traits. Characterization of quantitative trait loci is a proven method which can identify markers for use in selective breeding to provide a faster genetic gain through selection of proper candidate genes. In this regard, much work has been conducted on marker genes including (FASN, KLF3, KLF6, KLF15, ELOVL6, ABHD5, SIRT1, SIRT2, MTNR1A, SIX1, SIX4, MC4R, STAT3, and FTO), which have been identified with proven roles in adipogenesis, in cattle, pigs and other livestock [2,11,12,13,14,15,16,17,18,19,20,21].

Cholecystokinin (CCK) performs a key role in the storage of triglycerides in adipose tissue [22]. Moreover, the CCK is important for secretion of gastrointestinal peptide hormone from the I cells of the jejunum and duodenum [23]. This secretory function is stimulated by digesta flowing into the duodenum [24]. Recent findings have suggested that CCK is also involved in the brain-gut axis (vagus nerve, the hypothalamus, stomach and intestine) and is considered to regulate eating behaviors [25]. The main function of CCK is to regulate contraction of gallbladder and pancreatic secretions [26]. The physiology of CCK depends on cholecystokinin receptor (CCKR). The CCKR has two subtypes: CCKAR cholecystokinin A receptor (CCKAR) and CCKBR cholecystokinin B receptor (CCKBR). The CCKAR is primarily found in peripheral tissues, which regulates the gastrointestinal function and plays a key role in the regulation of the digestive system. [27]. The peripheral CCKAR regulates the CCK pathway through stimulation of vagus nerve, which in turn stimulates central nervous system (CNS) and regulates feeding behavior [28]. The CCKAR expression levels in the CNS is inversely proportional to the growth rate as it is a negative regulator of feeding behavior [29]. Therefore, it is an important regulator of feed intake and growth of animals. Moreover, previously it was found that polymorphism of CCKAR gene promoter affects fat deposition in humans [30]. Characterization of the CCKAR gene and its association with the growth and development of Qinchuan cattle has not yet been investigated. Thus, in the present study, the genetic polymorphism of the CCKAR gene was considered as a potential molecular marker for the improvement of the breeding of Qinchuan cattle.

2. Materials and methods

2.1. Ethical statement

All experiments were conducted in accordance with the guidelines of the China Animal Care Council and Northwest Agriculture and Forestry University, Yangling, China.

2.2. DNA sampling for phenotypic analysis

Totally, 228 female Qinchuan cattle (18–24 months old) were randomly selected for experiments from the farm of National Beef Cattle Improvement Center (NBCIC). The experimental animals were reared at the required environmental condition as per NBCIC standards [31]. Carcass quality characteristics were measured according to those described in the standard protocol of Gilbert and Gui [32,33] using a Sono-grader ultrasound machine (Renco, USA). The carcass quality traits including ULA and IMF % were recorded using ultrasound probe placing between the 12th and 13th ribs. The blood sample (5 ml) from the animals were carried out from jugular vein in anticoagulant tubes and immediately transferred to laboratory for DNA extraction. The extraction of

DNA from blood samples were conducted as described previously [34,35,36,37].

2.3. Polymerase chain reaction (PCR) amplification and genotyping

For the amplification of the 613 bp and 534 bp in the CDS (coding sequence) region of CCKAR (NC_037333.1) and Primer Premier 5 software (PREMIER Bio-soft International, CA, USA) was utilized for two pairs (reverse and forward) of primers were designed using (Table 1). This gene has 6 exons, the CDS region has a length of 2171 bp and the protein sequence has 427 amino acids (Fig. 1). The two target fragments were amplified using the KOD plus Neo Enzyme Kit (TOYOBO, Japan) as instructed by the manufacturer. Totally, 228 Qinchuan cattle were utilized for Genomic DNA as a PCR amplification template. PCR was conducted as; pre-denaturation at 94.0°C for 5 min, denaturation of 34 cycles of at 97.0°C for 30 s, annealing Tm x°C for 30 s (Table 1) and final extension at 72.0°C for 45 s. The polymorphisms were screened from PCR products using Sangon sequencing (Shanghai, China). The identification of SNPs were carried out using Seq Man (DNASTAR, Inc., USA) software.

2.4. Collection of tissue sample

The tissue samples in triplicates were collected from 7-day-old calves of Qinchuan cattle. Firstly, animals were dressed in a local slaughterhouse following the standard protocol i.e., animal stunning, exsanguinating, and skinning procedures. Secondly, to measure the relative expression of the CCKAR gene from tissues, 8 different tissues including (dorsal muscle, fat, heart, kidney, lung, liver, rumen and small intestine) were collected from Qinchuan calves. Finally, tissue samples were preserved immediately in liquid nitrogen and transferred to the laboratory for total RNA.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted separately from each tissue using TRIzol™ reagent (Invitrogen, Thermo-Fisher Scientific, Inc. USA). The NanoDrop ND-1000 spectrophotometer (peQLab, Erlangen, Germany) was utilized for the quality and concentrations of total RNA following the method as described by Raza et al. [12]. The Prime-Script™ RT Reagent Kit with gDNA eraser (Perfect Real Time, Takara) was utilized for cDNA and stored at –20°C for further analysis.

2.6. real-time PCR

The Sybr Premix EX Taq Kit (Takara, Dalian, China) was used to perform Quantitative RT-PCR. The prepared cDNA was used as a template for each tissue, and the gene-specific primers were used in a 20 µL reaction mix. Bovid GAPDH and β-actin were used as endogenous control. The SDS V 1.4.0 thermocycler 7500 system (Applied Biosystems, USA) was utilized for PCR. The cyclic conditions were followed as; preheating at 95°C for 5 min, denaturation of 34 cycles at 95°C for 30 s, annealing at 60°C for 30 s and 72°C for 30 s. Each reaction was performed in triplicate from each sample, and 2^{–ΔΔCT} method was applied for the relative expression levels of mRNA calculation as described previously [38].

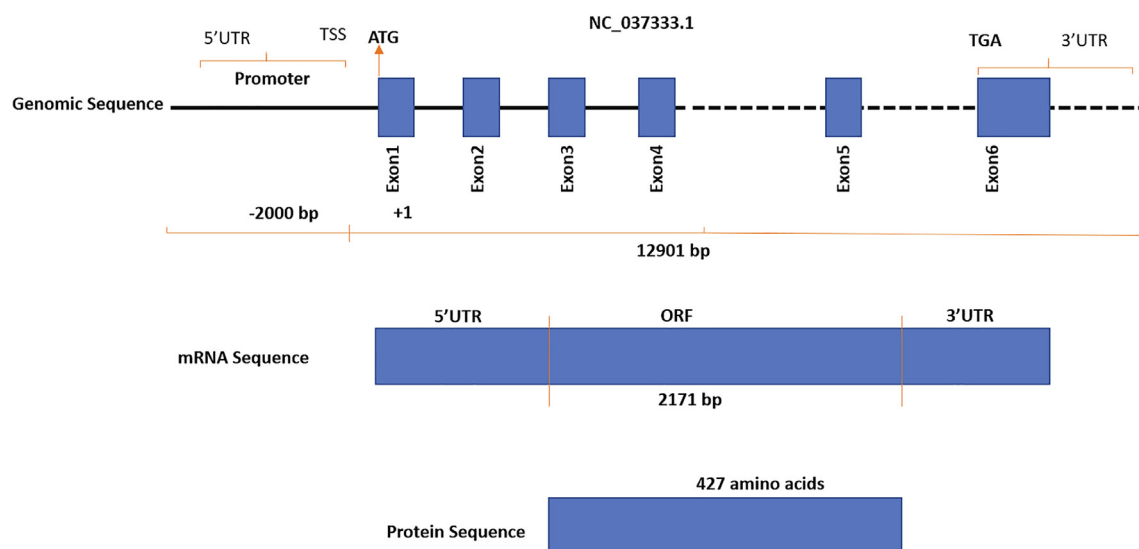
2.7. Data analyses

The SPSS 20.0 version (Chicago, USA) with the general linear model (GLM) was used for the analysis of associations between SNPs and selected carcass quality traits as previously described in published articles [39,40].

Table 1

Genotypic and allelic frequency of the CCKAR gene in Qinchuan beef cattle.

SNPs	Number	Genotype frequency			Allelic frequency		χ^2	PIC	Ne
g. 1463 C>G	228	CC 0.83 (172)	CG 0.16 (33)	GG 0.01 (22)	C 0.91	G 0.09	0.0876	0.1495	1.1944
g. 1532 T>A	228	TT0.89 (196)	TA0.11 (32)	AA0.00 (1)	T 0.94	A 0.06	0.7124	0.0990	1.1166
g. 1570 G>A	228	GG 0.92 (202)	GA 0.08 (25)	AA 0.00 (0)	G 0.96	A 0.04	0.3328	0.0712	1.0799
g. 1594 C>A	228	CC 0.86 (188)	CA 0.13 (39)	AA 0.01 (1)	C 0.93	A 0.07	0.7217	0.1284	1.1600
g. 1640 T>C	228	TT 0.34 (85)	TC 0.57 (102)	CC 0.14 (40)	T 0.62	C 0.38	8.6731	0.3601	1.8907
g. 1677 G>C	228	GG 0.89 (193)	GC 0.1 (33)	CC 0.01 (1)	G 0.94	C 0.06	0.9073	0.1070	1.1280
g. 1735 C>T	228	CC 0.89 (195)	CT 0.11 (33)	TT 0.00 (1)	C 0.94	T 0.06	0.0542	0.1028	1.1220

**Fig. 1.** Structure of the bovine CCKAR gene.

$$GLM (Y_{ijkm} = u + G_i = A_j + A_k + S_m + E_{ijkm})$$

GLM was calculated as described in the equation, where “ Y_{ijkm} ” represents the measurement of traits on each animal; u = overall mean for each trait; G_i = fixed effect associated with the j th genotype; A_j = fixed effect of the j th age; A_k = fixed effect due to the dam age; S_m = random effect with the m th sire; and E_{ijkm} = standard error”.

2.8. SNP calculation of allelic and genotypic frequencies

For all three SNPs the allelic and genotypic frequencies were calculated using the HWE through the chi square test in version 3.2 of Pop Gene software [41]. Fr genetic indicators of populations, such as PIC and gene heterozygosity (He) were measured as described previously [42]. Haploview (<http://analysis.bio.cn/myAnalysis.php>) [43] determined the haplotypes and the D' and r^2 linkage disequilibrium (LD). The $22^{-\Delta\Delta CT}$ was utilized for the calculation of relative expression levels of CCKAR mRNA as described previously [38]. The results are presented as mean and standard error, whereas $p < 0.05$ was considered statistically significant.

3. Results

3.1. SNP identification

Seven SNPs were identified at loci g. 1463 C>G; g. 1532 T>A; g. 1570 G>A; g. 1594 C>A; g. 1640 T>C; g. 1677 G>C; and g. 1735 C>T in the coding region of the bovine CCKAR gene. The genotypes generated by SNP1 was CC, CG and GG; SNP2 comprised TT and TA; SNP3 produced GG, and GA; SNP4 CC, CA and AA; SNP5 TT, TC and CC; SNP6 GG, GC and CC; SNP7 CC and CT (Table 1). The analysis of allelic and genotypic frequencies showed that all seven SNPs deviated from the HWE (Table 1 and Fig. 2, $p < 0.05$). SNP3, SNP2, SNP6 and SNP7 exhibited lowest PIC values, while reset of the SNPs showed moderate polymorphism ($0.25 < PIC < 0.50$) [44].

3.2. Linkage disequilibrium and CCKAR gene haplotype identification

As shown in Table 2 and Fig. 3, the high LD (D'/r^2) was between the SNP2 and SNP3 (1.000/0.001); SNP3 and SNP6 (1.000/0.002); SNP3 and SNP7 (1.000/0.002); and SNP3 and SNP5 (0.994/0.020). Moreover, a total of 21 haplotypes were found, however, the haplotypes with the frequency less than 3% were excluded and the

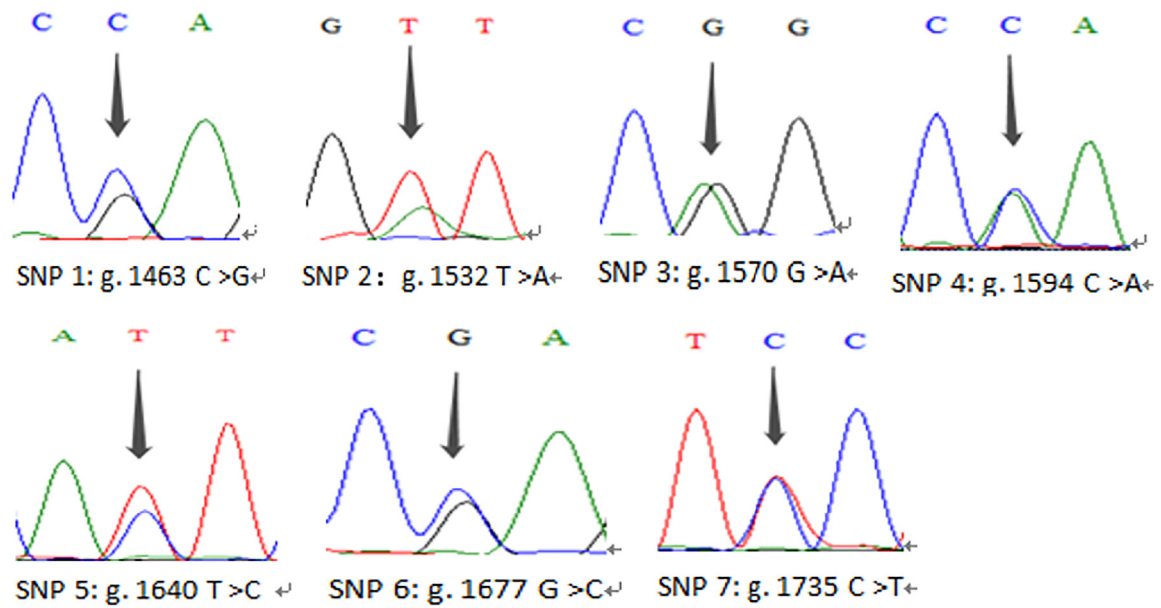


Fig. 2. SNPs in the coding sequence of the CCKAR gene in Qinchuan beef cattle.

Table 2
Linkage Disequilibrium tests among seven SNPs.

D'/r^2	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7
SNP1	0.428/0.070	0.780/0.002	0.948/0.746	0.929/0.037	0.870/0.503	0.786/0.448
SNP2		1.000/0.001	0.448/0.093	0.134/0.001	0.456/0.120	0.462/0.107
SNP3			0.564/0.001	0.994/0.020	1.000/0.002	1.000/0.002
SNP4				0.946/0.032	0.809/0.524	0.731/0.466
SNP5					0.252/0.005	0.262/0.006
SNP6						0.882/0.683

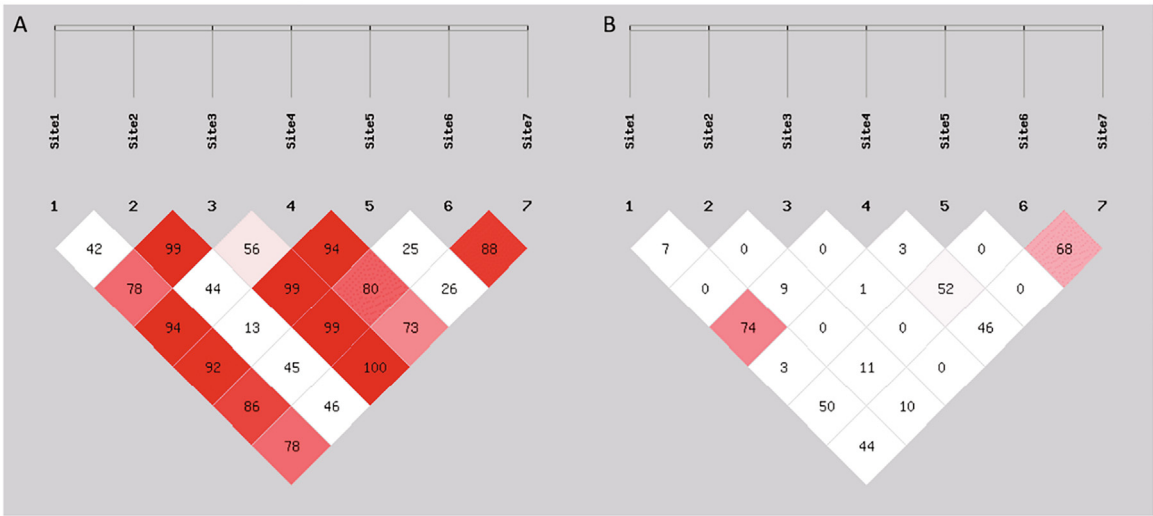


Fig. 3. Linkage disequilibrium between the seven SNPs in Qinchuan beef cattle. (A) Represents D' and (B) represents r^2 .

Table 3
Haplotypes frequency of the bovine CCKAR gene in Qinchuan beef cattle.

S. No	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	Frequency
1	C	T	A	C	T	G	C	0.310
2	C	T	G	C	T	G	C	0.540
3	C	T	G	C	C	G	C	0.327

Table 4
Genotype association of the CCKAR gene with body measurement and carcass quality traits in Qinchuan beef cattle.

Locs	Genotypes	BL (cm)	WH (cm)	HH (cm)	HW (cm)	CD (cm)	CC (cm)	BFT (cm)	ULA (m ²)	IP%
g.1463 C>G	CG	137.257 ± 2.799	122.136 ± 1.704	123.500 ± 1.378	40.000 ± 1.197	61.409 ± 1.561	169.151 ± 4.009	0.984 ± 0.055	51.177 ± 3.360	7.010 ± 0.322
	CC	135.456 ± 1.206	119.427 ± 1.195	122.982 ± 0.592	38.404 ± 0.529	58.606 ± 0.651	163.000 ± 1.889	0.924 ± 0.028	47.151 ± 1.311	7.059 ± 0.118
	GG	134.250 ± 4.250	118.500 ± 5.500	122.000 ± 3.000	38.500 ± 1.500	60.000 ± 2.000	168.500 ± 6.500	1.145 ± 0.045	60.845 ± 3.765	7.810 ± 0.260
g.1532 T>A	TA	135.652 ± 3.121	119.869 ± 1.986	123.782 ± 1.607	39.260 ± 1.123	59.087 ± 1.537	167.739 ± 3.900	1.049 ± 0.091	50.674 ± 3.259	7.323 ± 0.269
	TT	135.740 ± 1.172	119.845 ± 1.134	122.964 ± 0.572	38.583 ± 0.522	59.062 ± 0.646	163.567 ± 1.848	0.9218 ± 0.026	47.579 ± 1.308	7.023 ± 0.119
g.1570 G>A	GA	136.500 ± 3.142	121.625 ± 2.429	123.250 ± 1.659	38.687 ± 1.583	58.906 ± 1.696	159.750 ± 9.011	1.003 ± 0.071	50.606 ± 3.294	6.803 ± 0.581
	GG	135.666 ± 1.160	119.700 ± 1.100	123.039 ± 0.567	38.656 ± 0.504	59.078 ± 0.634	164.385 ± 1.688	0.930 ± 0.027	47.697 ± 1.291	7.080 ± 0.110
g.1594 C>A	CA	134.388 ± 2.685	120.611 ± 1.752	122.537 ± 1.423	39.000 ± 1.226	60.481 ± 1.738	164.814 ± 4.044	0.943 ± 0.052	48.222 ± 3.140	7.108 ± 0.339
	CC	135.838 ± 1.202	119.675 ± 1.166	123.069 ± 0.582	38.569 ± 0.525	58.787 ± 0.641	163.709 ± 1.869	0.927 ± 0.028	47.805 ± 1.335	7.038 ± 0.118
	AA	144.250 ± 14.250	125.000 ± 12.000	128.750 ± 9.750	42.000 ± 5.000	64.750 ± 6.750	182.000 ± 20.000	0.946 ± 0.455	54.270 ± 3.973	8.165 ± 0.095
g.1640 T>C	TC	135.331 ± 1.479	118.398 ± 1.633	122.521 ± 0.741	38.235 ± 0.633	58.865 ± 0.838	163.705 ± 2.187	0.918 ± 0.031	47.894 ± 1.613	6.996 ± 0.144
	TT	136.792 ± 1.778	121.835 ± 1.031	123.557 ± 0.816	39.300 ± 0.811	59.135 ± 0.924	163.385 ± 3.003	0.931 ± 0.045	46.685 ± 1.827	7.028 ± 0.209
	CC	134.315 ± 4.042	121.605 ± 2.779	124.552 ± 2.051	38.947 ± 1.771	60.052 ± 2.031	168.421 ± 6.209	1.062 ± 0.113	52.643 ± 5.584	7.561 ± 0.225
g.1677 G>C	GC	135.023 ± 3.335	121.119 ± 2.191	123.000 ± 1.861	39.476 ± 1.348	60.309 ± 2.108	165.904 ± 4.975	0.947 ± 0.077	48.630 ± 3.559	7.273 ± 0.339
	GG	135.627 ± 1.171	119.684 ± 1.139	123.073 ± 0.570	38.516 ± 0.519	58.866 ± 0.632	163.641 ± 1.832	0.929 ± 0.027	47.663 ± 1.313	7.031 ± 0.118
	CC	147.000 ± 8.621	121.000 ± 4.000	122.333 ± 3.333	41.666 ± 2.603	62.500 ± 2.291	174.666 ± 8.192	1.280 ± 0.380	58.773 ± 4.690	7.233 ± 0.782
g.1735 C>T	CT	135.409 ± 3.203	121.340 ± 2.100	123.159 ± 1.781	39.818 ± 1.330	60.772 ± 2.063	167.272 ± 4.937	0.979 ± 0.080	50.087 ± 3.693	7.315 ± 0.326
	CC	135.860 ± 1.183	120.248 ± 0.988	123.073 ± 0.574	38.541 ± 0.519	58.909 ± 0.632	163.781 ± 1.837	0.932 ± 0.027	47.858 ± 1.300	7.020 ± 0.119
	TT	130.166 ± 6.784	84.500 ± 35.200	121.166 ± 2.166	37.333 ± 3.756	56.000 ± 3.055	155.333 ± 5.238	0.823 ± 0.140	35.863 ± 10.678	7.516 ± 0.533

BL (Body length); WH (withers height); HH (Hip height); HW (Hip width); CD (chest depth); CC (chest circumference); BFT (Back fat thickness); ULA (Ultrasound loin area).

remaining three haplotypes with their respective frequencies are shown in Table 3.

3.3. Association of genotype with body measurement and carcass quality traits

Based on SNPs, the coding sequence of the bovine CCKAR gene association with carcass quality and body measurement traits of Qinchuan cattle is shown in Table 4. The genotype CG in SNP1 at loci g.1463 C>G showed highest association with WH, HW, CD and CCF, while the genotype TA at the very same loci was associated with BFT, ULA and IMF content in Qinchuan cattle. The cattle with genotype AA at loci g.1594 C>A in SNP4 exhibited highest body length (BL), wither height WH, HH, HW, CCF, and IMF. The cattle with genotype CC at loci g. 1677 G>C in SNP6 were associated with larger BL, HW, CCF, and BFT.

3.4. CCKAR gene expression profile in different tissues of Qinchuan beef cattle

The results of CCKAR relative mRNA expression levels in different tissues are shown in Fig. 4. The bovine CCKAR gene has a wide tissue distribution in Qinchuan cattle, with the highest expression in small intestine, adipose tissue, muscle and liver. The mRNA expression level in the other digestive system organs including reticulum, abomasum and omasum was moderate. A slight expression level of CCKAR mRNA was found in the large intestine, kidney and rumen.

4. Discussion

Body measures and carcass characteristics of cattle are affected by various factors which include animals age, environmental factors, conditions of management, nutrition, and genetics [3,18,45,46,47,48]. Selective breeding is an effective strategy for achieving sustainable improvement in these economically important traits, but it is time consuming to achieve genetic gain due to the longer generation interval. Genomic selection can help to increase the rate of improvement of traits and reduces progeny testing costs [49,50]. Assessments based on SNP genotypes can be calculated as soon as DNA can be obtained which allows early life selection in both sexes [51,52]. In the present study, a total

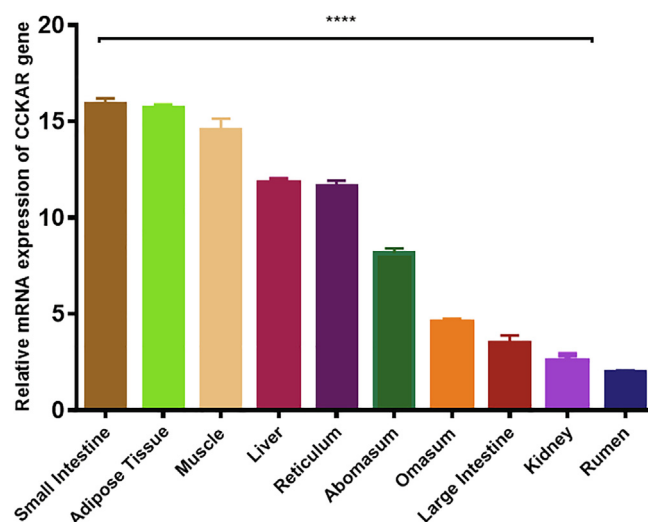


Fig. 4. Expression profile of the CCKAR gene in different tissues of Qinchuan beef cattle.

of seven SNPs were identified in the coding sequence region of the CCKAR gene. LD was analyzed between these SNPs. The results showed that the greatest LD (D'/r^2) was between the SNP2 and SNP3 (1.000/0.001); SNP3 and SNP6 (1.000/0.002); SNP3 and SNP7 (1.000/0.002); and SNP3 and SNP5 (0.994/0.020). The D' and r^2 are two most commonly used indicators for the prediction of LD. The D' is a normalized LD coefficient, which is more specific and useful for the prediction of LD [53,54,55]. Researchers agree that the latter indicator is most commonly used to measure the LD in pairs and is therefore considered less sensitive than D' for the measurement of allele frequencies [43,56]. When $r^2 > 0.33$, the LD is considered strong enough to be used for mapping [43]. Therefore, based upon these two indicators, there is a strong linkage between SNP2 and SNP3, and SNP3 and SNP6.

According to a previous research, polymorphism of the CCKAR gene in the promoter region significantly affected fat deposition in humans [30]. Moreover, previously, the role of CCKAR gene was only explored in peripheral tissues and in the regulation of gastrointestinal function [29]. The CCKAR gene SNPs identified in this study are causal variants that could be used for genomic selection of economically important traits [57]. Here in the present study, expression level of the CCKAR gene in small intestine, adipose tissue, muscle and liver was found to be higher. Thus, the CCKAR gene is considered an important regulator of feeding behavior, feed intake and growth in animals [28].

Ethics Statement

The China Council on Animal Care guidelines was used during while dealing with animals in all steps of experiments. Approval was further granted for all the experimental protocols by the Experimental Animal Management Committee (EAMC) of Northwest Agriculture and Forestry University, Yangling China.

Conflicts of Interest

The authors declare no conflict of interest.

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