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Molecular Characterization, Alternation Splicing, Expression Profile and Association Analysis with Carcass Traits of the Cattle *CMYA1* Gene

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Abstract: The Cardiomyopathy Associated protein 1 (CMYA1) is a actin binding protein which plays an important role in cardiac morphogenesis. In this study, researchers obtained the 6,272 bp mRNA sequence of the cattle *CMYA1* gene, including the full coding region and encoding a protein of 1,820 amino acids. The sequence was deposited into the GenBank nucleotide database (HQ111436.1). The human (NM_194293) and mice (NM_011724) homologues have a 77.31 and 82.23% identity with the cattle *CMYA1* gene, respectively. Tissue expression analysis showed that the cattle *CMYA1* gene was expressed exclusively in striated muscle tissue. Moreover, one deletion variant lacking 382 bp from exon 2 was detected by RT-PCR. Two synonymous SNP rs110346467 and SNP rs110575295 in the exon 2 detected, as restriction fragment length polymorphism showed genotype frequencies among 120 crossbred cattle. Association analysis showed that a SNP rs110575295 was significantly associated with the intermuscular fat percentage between 12th and 13th ribs ($p<0.05$). This study will lay the groundwork for the further investigations on the detailed function of CMYA1 in cattle models.

Key words: Association analysis, cattle, CMYA1, single nucleotide polymorphism, alternation splicing

INTRODUCTION

The *CMYA1* (Cardiomyopathy-Associated 1) gene, also called Xin which was initially identified using differential mRNA display from the developing chicken heart. Chick Xin (cXin) participates in a BMP-Nkx2.5-MEF2C pathway to regulating cardiac morphogenesis (Wang *et al*., 1996, 1999). Xin is localized at the adherens junctions of the intercalated discs in the heart and the myotendon junctions in skeletal muscle (Lin *et al*., 2005). Mammalian Xin colocalizes with N-cadherin in developing cardiomyocytes and with N-cadherin, connexin-43, filaminC, Vasodilator Stimulated Phosphoprotein (VASP) in the Intercalated Discs (IDs) of adult hearts (Otten *et al*., 2010). Interaction studies using co-immunoprecipitation, pull-down and yeast two-hybrid assays results revealed that mXin directly interacts with β-catenin. The β-catenin binding site on mice Xinα mapped to amino acid 535-636 (Choi *et al*., 2007). mXinα is able to bundle actin filaments and interact with β-catenin suggesting a role in linking the actin cytoskeleton to N-cadherin/β-catenin adhesion (Grosskurth *et al*., 2008). The expression of both cXin and mXin is regulated by the muscle transcription factor, MEF2C and the homeodomain transcription factor, Nkx2.5. The expression of mXinα in Nkx2.5 or MEF2C knockout mice embryos was drastically decrease to undetectable levels. On the other hand, the expression of mXin was up-regulated when mice were subjected to pressure-overload induced by cardiac hypertrophy (Lin *et al*., 2005). The myogenic transcription factors myogenic differentiation factor-D (MyoD) and myogenic factor-5 (Myf-5) are capable of transactivating Xin transcriptional activity. Moreover, Xin was significantly up-regulated during the early phases of skeletal muscle regeneration.

Hitherto, previous research on CMYA 1 has indicated that it functions in muscle tissues, relatively little is know concerning the cattle CMYA1. In this study, researchers cloned cattle CMYA1 cDNA and its alternative variant, analyzed tissue expression profile. Researchers, also detected its single nucleotide polymorphism and performed an association analysis in indigenous bos Mongolia×bos Simmental sire crossbred cattle. The present investigation was aimed at gaining an insight into the *CMYA1* gene, screening candidate genes for beef cattle breeding.

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MATERIALS AND METHODS

Animals blood and tissue samples: The experimental F3 population (n = 120 male animals) was produced by crossing six purebred. Simmental sire with no relationship F2 crossbred females (F1 50% indigenous bos Mongolia: 50% Simmental crossbred with purebred Simmental sire). All the animals were raised under normal management conditions according to the NY/T 815-2004 feeding standards in one farm. Average age at slaughter was 390 days. Animals were serially slaughtered at a commercial processing plant (Horqin Beef cattle Ltd., P.R. of China). Carcass measurements was taken according to criterion GB/T 17238-2008 of the cutting standard for fresh and chilled beef in China. The blood samples of the animals were collected. Ten different tissues including heart, gastrocnemius muscle, longissimus dorsi muscle, liver, spleen, lung, kidney, mammary gland, testicle and renal fat tissues were collected from two mature crossbred cattle, then immediately frozen in liquid nitrogen and then stored at -80°C for analysis.

Genomic DNA isolation, total RNA extraction and cDNA preparation: Genomic DNA was isolated from the blood samples of the 120 animals using a standard protocol (Sambrook *et al*., 1989). The total RNA was extracted from different tissues with a TRIzol® Reagent (Invitrogen, USA) following the manufacturer's instructions. Briefly, about 100-200 mg tissues were collected, placed in 2 mL of TRIzol reagent in a glass homogenizer, then homogenized immediately. The homogenate was then centrifuged at 12,000×g 5 min at 4°C. The aqueous phase was transferred into a clean tube, extracted by adding chloroform and then centrifuged at 12,000×g 15 min at 4°C. The resultant aqueous phase was saved and mixed with isopropyl alcohol to precipitate RNA. The RNA was collected by centrifugation at 7500×g 5 min at 4°C. The pellet was washed once with 75% enthanol, the final RNA preparations were resuspended in DEPC (Diethylene Pyrocarbonate-treated) water. In case, the samples were contaminated with genomic DNA, DNase I (Fermentas) treatment on the total RNA was carried out before first-strand cDNA synthesis. The quality of total RNA sample was evaluated by electrophoresis on 1.2% agarose gels stained with ethidium bromide and their concentrations were measured with the GENESYS 10S UV-Vis (Thermo Scientific). Total RNA was used to synthesize cDNA using RevertAid™ Premium Reverse Transcriptase (Fermentas) in a 20 μL reaction volume according to the manufacturer's instruction. Briefly, a mixture of 1 pg-5 μg total RNA, 50 pmol of random primer, 50 pmol of oligo (dT) primer and 1 μL dNTPs (10 mM each) was incubated at 65°C for 5 min to break the RNA secondary structure. The mixture was then transferred to ice for 2 min and then 4 μL 5×RT buffer, 20 U RNase Inhibitor and 200 U RevertAid™ Premium Reverse Transcriptase were added for a total volume of 20 μL. The RT mix was incubated at 25°C for 10 min followed 50°C for 30 min. The resulting cDNA was stored at -80°C until use.

Isolation and cDNA sequence analysis of the cattle *CMYA 1* gene: In an attempt to isolate the sequence encoding the cattle *CMYA 1* gene, the human mRNA sequence of CMYA1 transcript variant 1 (GenBank accession No. is NM_194293.2) was compared to non-human, non-mice ESTs database using highly similar sequences BLAST algorithm (http://www.ncbi.nlm.nih/gov/blast/). The highly conserved ESTs were assembled into a contig using the DNA Star program (Madison, WI, USA). The cattle EST which shared at least 80% identity to the corresponding human mRNA were selected to design gene specific primers (Table 1). The primers was synthetized by BGI Co. Ltd., in China. PCR amplifications were carried out in a 25 μL reaction mixture containing 1.25 μL (50 ng) of cDNA or DNA as template, 1 μL of each primer (10 μM), 0.5 μL dNTPs (10 mM), 2.5 μL of 10×PCR buffer with 1.5 mM $MgCl_2$ and 0.625 units of Taq DNA polymerase (CW, BioTech Co. Ltd., China) and 18.625 μL nuclease-free water. The PCR amplification profiles was as follows: 95°C initial denaturation for 5 min, 35 cycles of 94°C denaturation for 30 sec, 50-62°C annealing for 30 sec and 72°C extension for 30-90 sec (according the length of the target fragments), followed by a 10 min extension at 72°C. PCR were performed in the S1000 Thermal Cycler (Bio-Rad, singapone). The PCR products were purified with Gel Extraction Kit (Solarbio, China) and cloned into the pUCm-T vector (Sangon Biotech, China). Sequenced commercially using M13-forward nd M13-reverse primers (BGI Co. Ltd.,

Table 1: Primer pairs designed for the cattle *CMYA1* gene

Primer names	Primer sequences (5'-3')	Temperature (°C)	Product size (bp)
PF1	CCCGCAAGTTTGAGGAAG	55	882
PR1	CATTGGATAGATGCTCGTTTGT		
PF2	AGCCAGGTGCGGGTGAT	56	1418
PR2	ACGGGCTGCTCTTCCTG		
PF3	TCACAAGCAAGGCACG	54	880
PR3	AGGGAGTAGGCGGTCAG		
PF4	AGCATCATCCACGTTCC	51	671
PR4	CCTGCCATCACCTTCT		
PF5	TCCACGTTCCCCCACTGGACC	62	1150
PR5	TGTGGCCGAGGGTCGTGGTT		
PF6	CATCCGCTGGTTGTTCG	55	483
PR6	ACGGGCTGCTCTTCCTG		
GAPDHPF	ATGCTGGTGCTGAGTATGTG	56	605
GAPDHPR	AGTGTCGCTGTTGAAGTCG		

China). The Open Reading Frame (ORF) and the amino acid sequences were deduced with the program Seqman (DNAstar, Madison, WI, USA). The secondary structure was performed by online tools of the ExPASy website (http://cn.expasy.org/tools/). Multiple sequence alignments were carried out using the CLUSTALW 1.83 program (http://www.ebi.ac.uk/clustalw/) and the unrooted phylogenetic tree was constructed by DNAStar's LaserGene software (DNAstar, Madison, USA).

Tissue expression analysis of the cattle CMYA1 gene: The *CMYA1* gene specific primer pair PF6/PR6 (Table 1) was designed to detect the expression of cattle CMYA1 by RT-PCR. House-keeping gene *GAPDH* which amplification spans intron was used as an internal control to prevent DNA contamination. The negative control PCR was carried out with non-reverse transcription RNA as templates. The PCR reaction was carried out with the following cycling parameters: 95°C initial denaturation for 5 min, 30 cycles of 95°C denaturation for 30 sec, 51°C annealing for 30 sec and 72°C extension for 30 sec. A final extension was performed at 72°C for 10 min. The PCR products were visualized on 1% agarose gels stained with ethidium bromide and visualized with ultraviolet light.

SNP identification and allele frequencies: Using cattle genomic DNA, two parts of DNA sequence of CMYA1 in the exon 2 was obtained by the primer pair PF1/PR1 and PF6/PR6. The PCR reactions were performed as described earliar. The products were pooled and direct sequencing. The PCR restriction fragment length polymorphism (PCR-RFLP) were carried out by digests overnight 10 μL of PCR products with 20 U of Hin6I/ApaI (Fermentas) at 37°C. A total of 120 cattle DNA samples were genotyped and allele frequencies were determined, respectively.

Association analysis of the cattle *CMYA1* gene with carcass traits: The animals used in the association analysis of *CMYA1* gene were 120 cattle. Carcass traits were recorded including hot carcass weight (kg), marbling score, carcass length (cm), dressing percentage, longissimus dorsi area (cm^2), backfat thickness at the shoulder (cm), intramuscular fat percentage between 12th and 13th ribs (%), muscle colour score and loin pH. The association between different genotypes and carcass traits was performed with the least-squares method (GLM procedure, SAS version 8.0, SAS Institute, Inc.). The statistical model was assumed to be:

$$Y_{ij} = \mu + S_i + Y_j + e_{ij}$$

Where:

Y_{ij} = The observed value of different traits
μ = The least-square mean
S_i = The fixed effects of the genotypes
Y_j = The batch
e_{ij} = The random error.

Values are considered significant at $p<0.05$ and are presented as least square means±Standard Error

RESULTS AND DISCUSSION

PCR amplification and sequence analysis: The cDNA sequences amplified by primer pairs 1-6 and the cattle EST DV802652.1, DY138444.1, DY191701.1, DY051477.1, DY044036.1, EE371471.1, DV798947.1 were assembled into a contig of 6,272 bp and submitted to NCBI GenBank under the accession number of HQ111436.1 which including 143 bp a of the 5'-UTR, 5463 bp of the complete coding region and 666 bp of a 3'-UTR with a putative polyadenylation consensus signal (AATAAA). Primary structure analysis revealed that the molecular weight of the CMYA1 protein 194.8 kDa with an isoelectric point (pI) of 6.01. By BLAST with NCBI GenBank cattle genome the cattle of CMYA1 mRNA was mapped to chromosome 22 position 12,655K-12,665K bp which is near the loci Cysteine-Serine-Rich Nuclear Protein 1 (CSRNP1) and chemokine (C-X3-C motif) receptor 1 (CX3CR1). A phylogenetic analysis was done with nine vertebrates showed that the Danio rerio and Gallus gallus *CMYA1* genes form a separate cluster. Functional motif prediction revealed that the cattle CMYA 1 protein contained a proline-rich region, a helix-turn-helix motif, 8 significant and 7 insignificant actin binding sixteen amino acid repeat. The actin binding sixteen amino acid repeat has consensus sequence GDV(K/Q/R)(T/S/G)X(R/K/T)WLFE TXPLD. It was found that three Xin repeats are necessary and sufficient to bind the actin filament (Pacholsky *et al*., 2004) and the aminoterminus of human CMYA1 binds the EVH1 domain of Mena/VASP/EVL (Van der Ven *et al*., 2006). The gastrocnemius muscles injuryed mice model within 12 h were assessed by RT-PCR analysis, the result demonstrated that CMYA1 mRNA is robustly up regulated. The Xin is involved in the remodeling of the actin cytoskeleton of striated muscles during sarcomere assembly and cardiac morphogenesis. The current study suggests that Xin is one of the components at the adherens junction of cardiac muscle and its counterpart in skeletal muscle during sarcomere assembly and cardiac morphogenesis (Lin *et al*., 2005; Pacholsky *et al*., 2004; Sinn *et al*., 2002). Because of the similar sequence we predict that the cattle *CMYA1* gene also have the same function.

The cattle *CMYA1* gene gives rise to splice variant by alternative splicing: Unexpectedly, the PF5/PR5 specific primers PCR mixture presented two bands on electrophoresis (Fig. 1). A search with the cattle *CMYA1* gene mRNA sequence for genomic sequences using NCBI BLAST (http://www.ncbi.nlm.nih.gov) identified cattle genomic clones mapping exclusively to chromosome 22, indicating that *CMYA1* is a single copy gene. Only the repeat region showed similarity to a second gene *CMYA3*, encoding a protein researchers had previously designated Xin repeat protein 2. The sizes of these PCR products were 1150 bp (no-deletion variant 1) and 768 bp (variant 2). The sequence of the 768 bp product lacked 382 bp (3486-3867 bp is located in the variant 1) from the exon 2. To verify whether the exon deletion observed in the samples was caused by genomic CMYA1 alteration, researchers also amplified the region of the CMYA1 genomic DNA that included the splicing site. Due to the PF5/PR5 specific primers was not spans intron the negative control PCR was carried out with non-reverse transcription heat RNA as templates presented one band. The variants that were revealed by the expression analysis and implies that this variability arises by a alternative splicing. The two alternative splicing of the *CMYA1* gene was also presented by longissimus dorsi muscle and gastrocnemius tissue cDNA. The human *CMYA1* gene splicing events predict three Xin variants: XinA the full-length protein, XinB a carboxyterminally truncated variant and XinC an aminoterminally truncated variant. Unable to detect XinC on Western blots of normal human heart protein extracts but RT-PCR demonstrated the simultaneous expression of all three predicted variants in the normal human heart (Van der Ven *et al*., 2006). The mXinα k nockout mice model have been generated and appear to develop normally. However, mXinβ is up-regulated in the mXinα knockout hearts suggesting that mXinβ may partially compensate for the loss of mXinα during development (Gustafson-Wagner *et al*., 2007). A novel mice model in which the mice deficient in all Xin variants was generated which showed a very mild phenotype probably due to compensation by the structurally related XIRP2 (Otten *et al*., 2010). So, researchers predict that the Xin 16 unique repeat motifs may have a basic unit roles.

Tissue expression analysis of the cattle *CMYA1* gene: The RT-PCR was used to study the tissue expression of the cattle *CMYA1* gene. The negative control PCR was carried out with non-reverse transcription RNA as templates which presented no bands. Positive control PCR (GAPDH) in each tissue displayed a basically

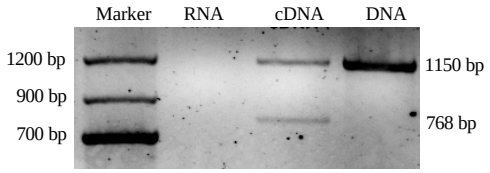

Fig. 1: The two transcript variants of the the cattle *CMYA1* gene; the two variants was directly sequencing with PF5 primers, the sequencing results was presented in supporting information

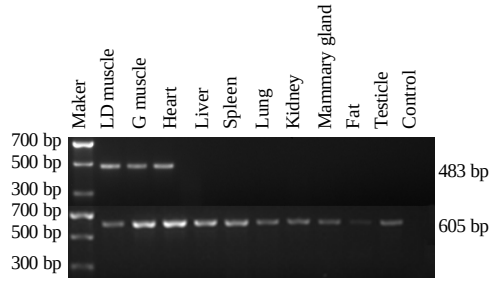

Fig. 2: Expression profiles of the cattle *CMYA1* gene; the tissue samples include longissimus dorsi muscle, gastrocnemius muscle, heart, liver, spleen, lung, kidney, mammary gland, fat tissues and testicle from mature croossbred Simmental. GAPDH acts as Positive Control PCR. M: Dmarker II ladder (CW, BioTech Co. Ltd., China)

identical signal. The gene was only expressed in heart and skeletal muscle tissues (Fig. 2). Thus, the result was consistent with the observation from humans and mice.

Genomic DNA amplification and SNP identification: Using genomic DNA, two parts of DNA sequence of CMYA1 in the exon 2 was obtained by the primer pair PF1/PR1 and PF6/PR6. The PCR-RFLP polymorphism were carry out by digests the PCR products with Hin6I/ApaI restriction enzyme, respectively. The SNP rs110346467 could be detected by restriction enzyme Hin6I, resulting in one fragment (518 bp) produced by allele T and two fragments (171 and 347 bp) produced by allele C (Fig. 3). The SNP rs110575295 could be detected by restriction enzyme ApaI restriction enzyme, resulting in one fragment (483 bp) produced by allele T and two fragments (252 and 231 bp) produced by allele C (Fig. 4). Genotype frequencies of the *CMYA1* gene determined by PCR-RFLP in 120 cattle was showed in the Table 2. In the tested cattle the SNP rs110575295 allele distribution showed that there are no CC genotype and genotype frequencies analysis revealed that the two SNP homozygous genestype was significantly excessed heterozygous which may causes by the cattle population genetic background and the small individual number of groups.

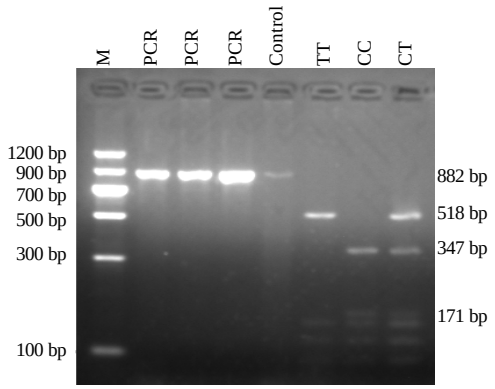

Fig. 3: SNP rs110346467 PCR-Hin6I-RFLP analysis was detected in exon 2 of the cattle CMYA1; the primer pair PF1/PR1 PCR products of CMYA1 in the exon 2 were pooled and sequenced confirm that the products has four Hin6I extinction enzymes cut site include SNP rs110346467. But, only SNP rs110346467 have polymorphism. The sequencing result was presented in the supplementary material. The control digestion were carried out in a 30 μL reaction mixture containing 2 μL of 10×Buffer, 10 μL PCR products, 18 μL nuclease-free water without Hin6I

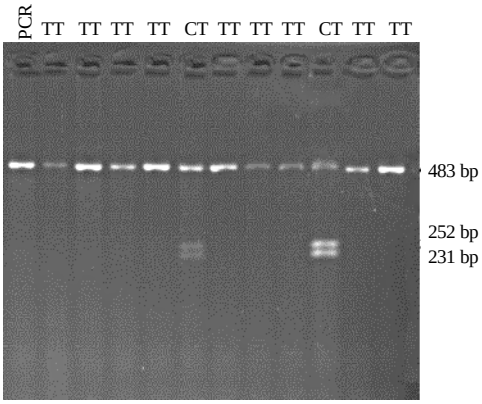

Fig. 4: SNP rs110575295 PCR-ApaI-RFLP analysis was detected in exon 2 of the cattle CMYA1; the primer pair PF6/PR6 PCR products of CMYA1 in the exon 2. Lane TT, CT and represent different genotypes, Lane PCR was a PCR products control

Association analysis of the cattle *CMYA1* gene with carcass traits: In the tested the SNP rs110346467 was not associated with any traits. Therefore, another SNP rs110575295 were further used for association analyses with the carcass traits. According to the association results (Table 3), there is significant associations between the polymorphism SNP rs110575295 and intramuscular fat percentage between 12th and 13th ribs ($p = 0.039$).

Table 2: Genotype frequencies of the *CMYA1* gene determined by PCR-RFLP in 120 cattle

SNP rs110346467			SNP rs110575295		
Genotype	No. of animals	Genotype frequency	Genotype	No. of animals	Genotype frequency
CT	33	0.275	CT	22	0.183
CC	58	0.483	CC	0	0.000
TT	29	0.242	TT	98	0.817

Table 3: Association of the SNP rs110575295 genotypes CMYA1 genotypes with carcass traits

Genotype	Intermuscular fatty acid QIB%
CT	7.570±0.5290
TT	7.904±0.4993

Association of the SNP rs110575295 genotypes with carcass traits in the experimental showed that TT genotype of the *CMYA1* gene bos taurus intermuscular fat percentage between 12th and 13th ribs were significantly excessed CT genotype of the *CMYA1* gene bos taurus

Combining the association analysis, expression and structure researchers predict a function of the cattle *CMYA1* gene for meat production.

CONCLUSION

Researchers first isolated the cattle *CMYA1* gene and performed necessary sequence analysis tissue expression profile analysis and one deletion variant of cattle CMYA1 were identified. This established the primary foundation for further research on the cattle *CMYA1* gene. The association results in the cattle population suggest that cattle *CMYA1* gene may be a candidate gene for inter muscular fatty acid performance and used as a genetic marker to improve this trait.

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