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Expression of Sheep CLPG in Different PRNP Genotypes

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Abstract: In order to investigate the possible relationship at the transcriptional level between CLPG and PRNP in sheep and the description of relationship of sheep reproductive with different PRNP genotype, the expression of CLPG gene in different tissues and different PRNP genotypes was determined using fluorescence quantitative PCR. The results showed CLPG has a tissue-specific gene expression as *CLPG* gene expression in cerebellumwas significantly higher than other organizations. However, genotyp specific expression was not detected in spite of the p-value in some tissues almost close to 0.05. Therefore, the researchers speculate that the expression of CLPG in different PRNP genotype may exist but the final conclusion need to be proved with the other genes which are related to growth trait in more different PRNP genotypes.

Key words: Sheep, CLPG, PRNP, fluorescence quantitative PCR, expression, China

INTRODUCTION

The callipyge phenotype in sheep is a muscular hypertrophy (Koohmaraie *et al*., 1995; Jackson *et al*., 1997). In an extensive survey of 19 muscles dissected from the right side of arcasses from normal and callipyge individuals (Jackson *et al*., 1997), the total weight of excised muscles from the pelvic, torso and thoracic limbs was greater in callipyge lambsthan in normally muscled half-sibs. Callipyge lambs exhibit several desirable production characteristics andmeat quality traits and exhibit superior feed efficiencies and lower daily feed intakes (Jackson *et al*., 1997). Enlargement of muscles in callipyge-expressing animals is primarily due to myofiber hypertrophy and the callipyge-responsive muscles exhibit larger average diameters (Carpenter *et al*., 1996). At present, researchers have investigated possible antagonistic associations between the PRNP locus and milk (De Vries *et al*., 2005), performance (Alexander *et al*., 2005) and growth traits (Isler *et al*., 2006; Vitezica *et al*., 2007) but evidence supporting antagonistic associations has not been found.

Transmissible spongi form encephalopathies such as scrapie in sheep is a significant hazard to livestock industries. Traditionally, culling of affected animals has been the standard method of scrapie controlin the sheep industry (Hunter *et al*., 1997; Andreoletti *et al*., 2006; Goldmann *et al*., 2006). Unfortunately, this method is not effective with prion diseases due to their long incubation time and unusual method of transmission. The PRNP haplo type encoding alanine, arginine and arginine (ARR) at the respective 136, 154 and 171 positions is associated with increased resistance to scrapie.

The objective of this study was to test for associations between PRNP genotypes such as ARQ/ARR and ARQ/ARHand expression of CLPG at mRNA level. The results would clarify the relationship between the PRNP expression and growth performance and the the possible roles of PRNP in sheep growth.

MATERIALS AND METHODS

Animals and tissue samples: In this study, researchers selected two types of prevalent heterozygous genotype healthy sheep in Gansu province of China, ARR/ARQ (four ewe animals) which isgenetically resistant to scrapie (Matuskova *et al*., 2003) and ARH/ARQ (four ewe animals) which genetically has intermediate susceptible to Scrapie (O'Doherty *et al*., 2002). The age of the animals ranged from 3-4 years old. All the sheep were negative to TSE by the PrP^{Sc} detection kit PlateliaBSE (Bio-Rad, Hercules, CA,USA) in brain. The animals were

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slaughtered and a sample from the same region of cerebrum, liver, spinal cord, ovary, brain stem, spleen, thalamus, kidney, cerebellum, heart and uterus were aseptically taken from each animal. All tissues were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction was performed.

RNA extraction and cDNA synthesis: Total RNA was extracted from 100 mg of tissue of eachsample using the TRIZOL Reagent (Invitrogen, Vienna, Austria) and treated with RNase-free DnaseI (TaKaRa, Japan) to remove possible contaminating DNA. The purity of the total RNA was estimated by the OD_{260}/OD_{280} absorbance ratio (1.9-2.0). Measurement of RNA concentration was conducted at OD_{260} nm on a spectrophoto meter (GE, USA). Constant amounts of 1000 ng of RNA were reverse transcribed to cDNA using 200 units of the reverse. Transcription system SS III (Invitrogen, Vienna, Austria) according to the manufacturer's instructions. Six randomly chosen control samples without transcriptase (RT-negative) were used as controls for RT step.

Construction of recombinant plasmid: The fragments of 129 bp target gene were amplified by the primers: 5'-TGTCCTGGTCTATTTTCGGG-3' (forward); 5'-GAATTGGAAGGCTTGGAGGT-3'(reverse)inreactionof 25 μL volume containing 1 μL of the above cDNA, 1 μL of each primer (10 pmol L^{-1}), 12.5 μL of Ex Taq mix (Takara, Japan) and 9.5 μL of sterile water. PCR reaction was conducted under the following conditions; an initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec, 72°C for 30 sec and a final extension at 72°C for 8 min (Bio-Rad thermal cycler, USA). The PCR products were analyzed by electrophoresis on 1.5% agarose gel.

In order to eliminate artifacts that could arise during the PCR process, ten PCR reactions were set up for each sample together with controls (no cDNA). PCR bands of interest were cut from the gel and purified using a commercial kit (E.Z.N.A gel extraction kit, Promega). The plasmid was constructed by cloning the PCR product into the pGEM-T easy vector (Promega) according to manufacturer's guidelines and were transformed into competent DH5α cells.

Quantitative RT-PCR: The ARQ plasmid DNA was quantified by absorbance at OD260 nm on a spectrophoto meter and the copy numbers were calculated. For standard curve acquisition, six 10-fold serial dilutions of plasmid DNA from 10^3-10^8 molecules were prepared and quantified by real-time PCR of CLPG gene inserts using Light cycler 480 II (Roche).

Six plasmid DNA standards cDNA of biological sample was analyzed in triplicate in a total reaction volume of 25 μL containing 1.0 μL plasmid DNA or 1 μL of the above cDNA (equivalent to 50 ng of reverse-transcribed total RNA), 12.5 μL of Platinum SYBR Green qPCR Super Mix-UDG 12.5 μL, 2.0 μL of $MgCl_2$ (50 mM), 0.5 μL of ROX, 1.0 μL of BSA (1 mg mL^{-1}), 1.0 μL of each primer (10 pmol L^{-1}), 7.5 μL of sterile water. Reactions were run on Light cycler 480 II using the following conditions; 50°C for 2 min, 95°C for 2 min and 40 cycles at 95°C for 15 sec, 55°C for 30 sec and elongation at 72°C for 20 sec. For each experiment, a non-template reaction was included as negative control. The specificity of the PCR reactions was confirmed by size verification of the amplicons in a conventional agarose gel.

Analysis of PRNP expression in tissue samples: CLPG gene expression in tissue samples were tested in triplicate and calibrated by the threshold values as described above and quantified using the absolute standard curve.

Statistical analysis: Data were analyzed by SPSS software (Statistical Package for the Social Sciences, Version 17.0 for Windows; SPSS Inc., Chicago, IL, USA). ANOVA method was applied to analyze difference in mRNA expression between all tissues of ARR/ARQ and ARH/ARQ genotype sheep, an independent sample t-test was used to analyze differences inmRNA expression between same tissue of ARR/ARQ and ARH/ARQ genotype sheep.

RESULTS AND DISCUSSION

Quantification of *CLPG* gene expression: The accuracy and reliability of CLPG real-time quantitative PCR were confirmed by the use of standard curve based on plasmid which resulted in a high linearity assay ($r^2 = 0.999$) over the six recombinant standard DNAs from 10^3-10^8 copies (Fig. 1). The linear regression equation was:

$$Y = 4.372X+45.26$$

Effect of PRNP genotypes on CLPG expression: Comparison of CLPG transcript levels between same tissue of ARR/ARQ and ARH/ARQ genotype sheep showed that the expression levels were not significantly different in ARR/ARQ and ARH/ARQ sheep ($p>0.05$).

Tissue-specific expression of CLPG transcripts: Expression levels of CLPG in different organs were examined in sheep of ARR/ARQ and ARH/ARQ genotypes and varied significantly. The data showed that

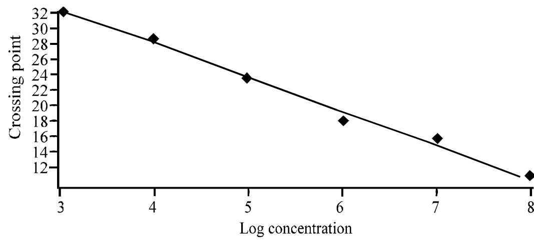

Fig. 1: A representative standard curve obtained the starting copy numbers of recombinant plasmid. Six dots are calibration dilutions (10^3-10^8 copies) of plasmid

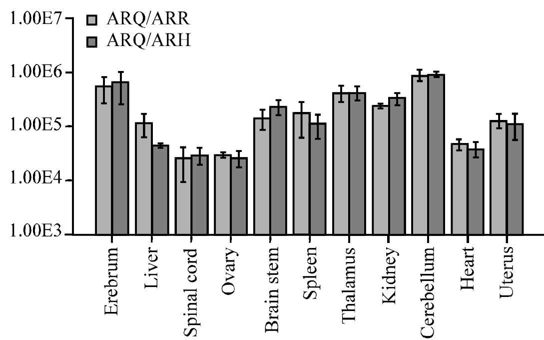

Fig. 2: CLPG mRNA copies in 100 μg of total RNA in different tissues of ARR/ARQ and ARH/ARQ sheep. Error lines indicate SE

CLPG expressed incerebellum was significantly higher ($p<0.05$) than other organs measured. CLPG expressed in cerebrum was also significantly higher ($p<0.05$) than spleen, brain stem and kidney (Fig. 2).

At present, the relationship of sheep PRNP genotypes between growth traits have been reported for many researchers. Someone reported there were significant effects for birth weight: Scottish blackface lambs heterozygous for ARQ hada higher birth weight than ARQ/ARQ lambs (3.52 vs. 3.48 kg) (Sawalha *et al*., 2007). In Dorset lambs, carriers of the VRQ allele were 0.6 kg>ARQ/ARQ lambs (Tonguea *et al*., 2006). Evidence for significant associations between PRNP genotype and weaning weight was also reported (Brandsma and Janss, 2004).

The effect in the Texel reflectedan advantage of about 1 kg in weight at 135ht day for VRQ carriers over ARQ/ARQ. German Black-headed mutton sheep heterozygous for the ARR allele grew more slowly than non-ARR animals. In the Swaledale breed, ARR carriers were heavier than non-ARR carriers at weaning but only the difference between the ARR heterozygotes and the non-carriers was significant (Sweeney and Hanrahan, 2008). The effects on slaughter weight in showed that heterozygous AHQ lambs were heavier at slaughter than non-AHQ carriers and ARQ homozygotes were lighter than either ARQ heterozygous animals or non-ARQ animals (Sawalha *et al*., 2007). There was also report showed a genetic association between scrapie susceptibility and lean tissue growth rate (Hunter, 1997). With regard to muscle depth, German black-headed mutton animals carrying the ARR allele had a smaller muscle depth than non-ARR animals (De Vries *et al*., 2004; Hanrahan *et al*., 2008).

CONCLUSION

The study shows that there was association between sheep PRNP genotype and some economic traits. As an important economic traits in sheep, analysis of *CLPG* gene expression levels in different sheep PRNP genotype had important significance. The results showed that comparison of CLPG transcript levels between same tissue of ARR/ARQ and ARH/ARQ genotype sheep showed that the expression levels were not significantly different in ARR/ARQ and ARH/ARQ sheep ($p>0.05$). This conclusion were different with the expected but the p-value in some tissues almost close to 0.05, the expression of CLPG in different PRNP genotype may be exist but need to be proved with the other genes which related to growth trait in more different PRNP genotypes. In addition, the researchers found that the expression levels of CLPG in different organs varied significantly: CLPG expressed incerebellum was significantly higher than other organs measured and expressed in cerebrum was also significantly higher than spleen, brain stem and kidney. The data will be helpful to understand *CLPG* gene expression features.

To the knowledge, quantitative analysis of CLPG transcription levels in different PRNP genotypes of sheep and the discussion of relationship between PRNP genotypes and CLPG transcription levels were first reported which will provide new ideas for future research of relationship of *PRNP* genes and economic traits from a new perspective.

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