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Selection and validation of suitable reference genes in skin tissue of Liaoning cashmere goat during hair follicle cycle

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ABSTRACT

In quantitative real-time PCR assay, the appropriate reference genes are essential to reduce the potential errors in quantification of gene expression. To date, there is not a well-defined and validated set of reference genes for skin tissue of cashmere goat. In the present work, based on the geNorm and NormFinder procedures, the expression stabilities of eight candidate housekeeping genes were assessed in skin tissue of Liaoning cashmere goat during hair follicle cycle including *18S*, *ACTB*, *B2M*, *GAPDH*, *TBP*, *UBC*, *YWHAZ*, and *SDHA*. Both geNorm and NormFinder identified *SDHA*, *YWHAZ* and *UBC* as the most stable genes in skin of Liaoning cashmere goat. Also, we further validated the suitability of *SDHA*, *YWHAZ* and *UBC* in combination as references in skin of Liaoning cashmere goat via detecting the relative expression of *TGFβR2*, *BMP2*, *MSX2* and *Hoxc13* as target genes. Significant differences were revealed in the relative expression of *TGFβR2*, *BMP2*, *MSX2*, and *Hoxc13* at anagen and telogen when a combination of *SDHA*, *YWHAZ* and *UBC*, versus single *SDHA* were used as reference genes. These results suggested that the combined use of three genes (*SDHA*, *YWHAZ*, and *UBC*) as references would be more reliable than that of a single *SDHA* for Q-PCR data normalization in skin tissue of Liaoning Cashmere goat. Therefore, we strongly recommended that these three genes can be used as a combined reference for normalization of gene expression in future longitudinal studies on skin of Liaoning cashmere goat associated with import cashmere traits.

1. Introduction

Liaoning cashmere goat, an excellent native breed, is found in the Buyun Mountains of Liaotung Peninsula at altitudes ranging from 500 to 1340 m (approximately 121°20′–125°40′ E, 30°20′–40°10′ N). It is of great economic importance to local people in that it forms a major source of household income for residents (Bai et al., 2012). Although, the main use of this breed is cashmere, it was demonstrated that this breed has a relatively greater cashmere fiber diameter with an average of 16.78 μm (adult male) and 15.87 μm (adult female) (Jiang et al., 2011). The thinner fibers would bring a higher economic gain for cashmere producers (Li et al., 2005).

In order to reveal molecular regulatory mechanism of cashmere growth, it would be important work for us to elucidate the expression characteristics of the genes, which were involved in hair follicle activity and fiber growth, in skin tissue during different stages of hair follicle cycle. Quantitative real-time PCR (Q-PCR) is rapid and reliable method for analyzing expression levels of interest genes in various tissues

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and organs (Yadav et al., 2012). However, an adequate normalization for gene expression data is essential to correct the differences in starting material, mRNA quality, enzymatic efficiency, and the presence of PCR inhibitors (Bustin, 2002; Imbeaud et al., 2005; Mehta et al., 2010). Among several strategies proposed for achieving this purpose (De Boever et al., 2008; Ginzinger, 2002; Huggett et al., 2005), the use of housekeeping gene as reference was commonly accepted and frequently used in normalization procedure of Q-PCR expression data (De Jonge et al., 2007). From a theoretical point of view, optimal housekeeping genes as references in Q-PCR normalization should be expressed stably with consistent and repeatable levels in all analyzed samples irrespective of tissue type and physiological state (Maccoux et al., 2007; Suzuki et al., 2000). Unlike expected, the expression of some housekeeping genes was shown to be varying considerably in different tissue type, developmental stage, and environmental conditions (Janovick-Guretzky et al., 2007; Schmittgen and Zakrajsek, 2000; Zheng and Sun, 2011). On the other hand, it was demonstrated that the use of a single housekeeping gene as reference appears to be insufficient, and normalization for Q-PCR data using multiple genes was recommended by many researchers (Pfaffl et al., 2004; Vandesompele et al., 2002; Varshney et al., 2012). Therefore, the preliminary evaluation and selection of candidate housekeeping genes as references have become rather important in the normalization of Q-PCR data (Hembruff et al., 2005; Huggett et al., 2005).

Seasonal hair follicle activity and cashmere fiber growth is a complex physiological process in cashmere goat (Wu et al., 2012). All mature hair follicles undergo a growth cycle consisting of early anagen, anagen, catagen and telogen phases (Stenn and Paus, 2001). For analyzing the expression level of genes involved in the cycle of skin hair follicles, it is significant to identify suitable housekeeping genes as references that are expressed stably among samples collected from skin tissue at various stages of hair follicle cycle. To date, there is no well-defined and validated set of reference genes for skin tissue of cashmere goat during hair follicle cycle. Therefore, the aim of the present work was to identify suitable references for studying gene expression in skin tissue of Liaoning cashmere goat.

2. Materials and methods

2.1. Sample, RNA extraction and cDNA synthesis

Six adult female individuals were selected from Liaoning Cashmere goat. Using sterile scalpel blades, skin tissue biopsies of about 1 cm^2 were harvested from the side of body in June (early anagen), October (anagen), January (catagen) and March (telogen). The goats sampled for skin tissue were clinically healthy, and avoided narrow genetic relationships. All experiments were conducted under the approval of the Animal Experimental Committee of Shenyang Agricultural University. The collected samples were immediately put into Sample Protector (TaKaRa, Dalian, China). Subsequently, they were transported to laboratory in low temperature environment and frozed at −80 °C. Total RNA was extracted from different samples with the RNAiso reagent kit (TaKaRa, Dalian, China). The integrity and quality of the RNA was checked by 1.5% agarose gel electrophoresis with ethidium bromide staining. The isolated RNA was treated with DNase I (TaKaRa, Dalian, China). The purity and quantity of the extracted total RNA were further assessed with ultraviolet spectrometer. Using M-MuLV cDNA Synthesis Kit (Sangon, Shanghai, China), the synthesis of first strand cDNA was carried out for each sample with 1 μg of total RNA.

2.2. Gene selection, primer design and Q-PCR

The *ACTB* or *GAPDH* was previously used as a single reference respectively in the gene expression analysis of skin tissue of cashmere goat (Su et al., 2009; Jin et al., 2011; Wu et al., 2012). Six additional genes, namely *18S*, *B2M*, *TBP*, *UBC*, *YWHAZ* and *SDHA*, were not typically used in skin tissue related experiments of cashmere goat. However, they were explored as candidate housekeeping genes as references in the skin tissue of other species such as mouse (Turabelidze et al., 2010) and dolphin (Spinsanti et al., 2006). These genes belong to different classes and their products play different roles in cellular function reducing the chance of potential co-regulation (Table 1). Additionally, the *TGFβR2*, *BMP2*, *MSX2*, and *Hoxc13* genes were also used as target genes to validate the identified optimal references for normalization of Q-PCR data in our study. These four genes were shown to play key roles in the regulation of skin hair follicle cycle (Stenn and Paus, 2001; Su et al., 2009; Wu et al., 2012). Thus, a total of 12 genes were used in the present work including eight candidate housekeeping genes and four target genes. Using the primer premier 5.0 software (http://www.premierbiosoft.com), the primers for the 12 genes were designed with the amplicon size in the range of 100–200 bp, and the information is shown in Table 1. The specificity of the primers was tested by challenging them on the first-strand cDNA obtained from this work using agarose gel electrophoresis (1.5%) with subsequent sequencing analysis.

The Q-PCR reactions were performed in a LightCycler 480 Real Time PCR system (Roche Diagnostics, Germany) with SYBR Green I assay. The 10-fold dilution of cDNA obtained from skin tissue total RNA was prepared, and 6-point standard curve was made for each housekeeping gene under analysis. A 20 μL reaction final volume was used consisting of 2 × SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) 10 μL, each of forward and reverse primers (10 μ*M*) 0.4 μL, first-strand cDNA 2.0 μL, and PCR grade water 7.2 μL. The cycle conditions were set as follows: initial template denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 15 s, 52–62 °C (Table 1) for 10 s, and 72 °C for 15 s. The last cycle was followed by a melting curve analysis ranging from 56 °C to 95 °C with a ramp speed of 0.5 °C per 10 s, to confirm that a single product was amplified for each primer set. Each reaction was run in triplicate, and the mean value was used in further data analysis. A blank control without cDNA template was incorporated in each assay. Using the given slops from the instrument' software, the Q-PCR efficiency (E) was calculated with the equation: $E = 10^{(-1/\text{slope})}$, and found to be close to two for each gene analyzed.