Selection and validation of suitable reference genes in adipose tissue of Jianzhou Da’er Goat (Capra hircus)

Guangjie Xie\textsuperscript{a,b,c}, Lu Tang\textsuperscript{a,b}, Yaqiu Lin\textsuperscript{b,c}, Mingfeng Jiang\textsuperscript{b,c}, Qing Xu\textsuperscript{a,b,c}, Jiangjiang Zhu\textsuperscript{a,b}, Qingyong Meng\textsuperscript{d} and Yong Wang\textsuperscript{a,b}

\textsuperscript{a}Key Laboratory of Sichuan Province for Qinghai-Tibetan Plateau Animal Genetic Resource Reservation and Exploitation, Southwest Minzu University, Chengdu, People’s Republic of China; \textsuperscript{b}Key Laboratory of Qinghai-Tibetan Plateau Animal Genetic Resource Reservation and Utilization, Ministry of Education, Southwest Minzu University, Chengdu, People’s Republic of China; \textsuperscript{c}College of Animal & Veterinary Sciences, Southwest Minzu University, Chengdu, People’s Republic of China; \textsuperscript{d}State Key Laboratories for Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing, People’s Republic of China

ABSTRACT

Reverse transcription quantitative real-time PCR (RT-qPCR) is a common and high-efficiency technique for detecting the mRNA levels of genes where suitable reference genes were introduced to normalize the data of RT-qPCR. However, little is known about the suitable reference genes in the adipose tissue of Jianzhou da’er goat. Here, six housekeeping genes, including the ACTB, ALAS1, EIF3K, HSP90, RPLP0, and UXT, were selected as candidate reference genes, while the FASN, HSL, LPL, and PID1 were used as the target genes for the validation of the suitability of identified reference genes. After detecting the expression levels of selected genes, the geNorm, NormFinder, and RefFinder softwares were applied to analyze the obtained data of candidate reference genes. The suitability analysis showed that the HSP90 was the most stable candidate reference gene, followed by the ALAS1 and RPLP0 genes. The validation experiment not only verified that the HSP90 was more stable than ACTB as the reference gene, but also confirmed that the HSP90 alone and a combination of HSP90, ALAS1, and RPLP0 could play the same roles in the normalization of target genes. Therefore, we strongly suggested that the HSP90 alone and the combination of three genes (HSP90, ALAS1, and RPLP0) could be used as the suitable reference for normalizing gene expression data in adipose tissue of Jianzhou da’er goat.

Introduction

Adipose, as main energy-storage tissue, has been proved to be involved in many metabolic processes and regulated by the adipose-related genes (Chilliard et al. 2000; Farmer 2006). Over the last decades, researchers have found that adipose tissue made a difference to the meat quality, where fatty acids influenced firmness and flavour of meat (Wood et al. 2004). Thus, investigating the features of adipose-related genes can help us to understand the formation of adipose tissue and further improve meat quality.

It is basic to detect the mRNA levels of genes in tissues for acquiring their features (Le Roch 2003). There are many techniques to attain the expression levels of genes, but quantitative real-time polymerase chain reaction (RT-qPCR) has been becoming the most common and reliable one (Heid et al. 1996). In order to make expression levels of target genes more accurate, reference genes like housekeeping genes, including the ACTB, GAPDH, and 18S, are always introduced to normalize the obtained data from RT-qPCR (Huggett et al. 2005; Bustin et al. 2009). Also, it was turned out that the HSP90 could be the suitable reference gene in visceral adipose tissues of the native Iranian goat (Capra hircus), while in subcutaneous adipose tissues was the ALAS1 (Najafpanah et al. 2013). The UXT, EIF3K, and RPLP0 were the most stable reference genes in bovine (Bovine) subcutaneous and alpine caprine (Capra hircus) omental adipose tissues (Bonnet et al. 2013). For Jianzhou da’er goat (Capra hircus), a Chinese meat-producing goat breed with many outstanding traits (Emu et al. 2014), however, the suitable reference genes in its adipose tissue for the normalization of gene expression remained unclear. It was also reported that the combination of reference genes did normalize the gene expression better than a single one (Vandesompele et al. 2002; Varshney et al. 2012). Therefore, it is necessary to select and validate appropriate reference genes to carry out the normalization for the gene expression in adipose tissue of Jianzhou da’er goat.

In the present paper, we selected six housekeeping genes involved in different biological processes as candidate reference genes, including the ACTB, ALAS1, EIF3K, HSP90, RPLP0, and UXT. Also, the FASN, HSL, LPL, and PID1, which were found to play crucial roles in lipid metabolism processes,
were considered as the target genes (Wakil 1989; Goldberg 1996; Schweiger et al. 2006; Yin et al. 2019). Subsequently, the expression levels of selected genes were detected by the RT-qPCR technique in abdominal and dorsal adipose of Jianzhou da’er goat, respectively. The stability of six candidate reference genes was ascertained by the geNorm, NormFinder, and RefFinder softwares, and the optimal number of reference genes was analyzed by the geNorm as well (Vandesompele et al. 2002; Andersen et al. 2004; Xie et al. 2012). Taken together, this paper aimed to identify the suitable reference genes for normalizing the data of RT-qPCR to analyze the relative mRNA levels of genes in adipose tissue of Jianzhou da’er goat.

Materials and methods

RNA extraction from adipose tissue and the synthesis of single-stranded cDNA

Six 24-month-old healthy adult wether individuals from Jianzhou da’er goat breed (Capra hircus) were selected from Sichuan Jianyang Big Brother Animal Husbandry CO., LTD. All experiments were carried out under the approval of the Animal Care and Use Committee of Southwest Minzu University. Approximately 1 cm³ abdominal and dorsal adipose tissues of individuals were harvested and put into RNA enzyme-free centrifuge tubes of 1.5 mL with tin foil, respectively. Subsequently, they were carried to our laboratory in a low-temperature environment. Total RNA was extracted from 100 to 200 mg abdominal and dorsal adipose of Jianzhou da’er goat using 1 mL TRizol reagent (Takara, Dalian, China) according to the manufacturers’ instructions (Rio et al. 2010). Briefly, after adding 0.2 mL of chloroform to every RNA sample, the samples were mixed vigorously for 15 s, incubated on the ice for 10 min, and centrifuged at 12,000 rpm for 15 min at 4°C. Then the supernatant of every sample was extracted to a new RNA enzyme-free centrifuge tubes of 1.5 mL and mixed softly after adding isovolumic isopropanol. The samples were placed at room temperature for 10 min and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded and the RNA precipitate was washed once with 75% ethanol (in DEPC-treated water). After discarding supernatants again, the RNA precipitate was air-dried and then dissolved in 20 μL DEPC water. The quality of RNA was checked by 1% agarose gel electrophoresis. Also, based on the total RNA of 1 μg, the cDNA was synthesized by the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China).

Selection of candidate reference genes along with target genes

Based on related literatures, six housekeeping genes playing different roles in multiple biological functions (Table 1), including the β-actin (ACTB), aminolevulinate delta-synthase 1 (ALAS1), eukaryotic translation initiation factor 3K (EIF3K), heat shock protein 90 (HSP90), ribosomal protein large P0 (RPLP0), and ubiquitously expressed transcript (UXT), were selected as candidate reference genes for reducing the possibility of gene co-regulation. The ACTB was the most frequently used reference gene in human adipose tissue, while the HSP90 and ALAS1 were found to be the most stable reference genes in adipose tissue of the native Iranian goat (Capra hircus), and the UXT, EIF3K, and RPLP0 were suggested to be the most stable reference genes in bovine (Bovine) subcutaneous and alpine caprine (Capra hircus) omental adipose tissue (Gabrielson et al. 2005; Bonnet et al. 2013; Najafpanah et al. 2013). Additionally, four lipid metabolism-related genes, including the fatty acid synthase (FASN), hormone-sensitive lipase (HSL), lipoprotein lipase (LPL), and phosphotyrosine interaction domain containing 1 (PID1), were selected as the target genes and their main functions were listed in Table 1 (Wakil 1989; Goldberg 1996; Schweiger et al. 2006; Yin et al. 2019).

Primers design and RT-qPCR assay

The primers for the used genes spanning the introns were designed using the Primer Premier 5.0 software and estimated by the Blast tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in NCBI with the related information listing in Table 1. For checking the primer specificity, we built their melt curves and standard curves by the RT-qPCR technique. The 1000-fold dilution of the initial cDNA (50 ng/μL) obtained from adipose tissue total RNA was prepared, and we diluted it to the 10⁴, 10⁵, 10⁶, 10⁷, 10⁸, and 10⁹-fold. The total volume of RT-qPCR was 10 μL: iQTM SYBR® Green Supermix (BIO-RAD) 5.0 μL, template cDNA 0.8 μL, Primer F (1.0 μmol/L) 0.5 μL, Primer R (1.0 μmol/L) 0.5 μL, and ddH2O 3.2 μL. The work conditions were set as follows: initial cDNA denaturation at 95°C for 10 min, followed by 39 cycles of denaturation at 95°C for 10 s, 55°C–63°C for 20 s (Table 1), and 72°C for 45 s.

Stability evaluation and optimal number analysis of candidate reference genes

The geNorm, NormFinder, and RefFinder softwares were used to ascertain the stability of six candidate reference genes according to the authors’ instructions (Vandesompele et al. 2002; Andersen et al. 2004; Xie et al. 2012). In the geNorm, which is a part of Biogazelle’s qbase + software, the average expression stability M is used to evaluate the stability of candidate reference genes. During the operation process of the geNorm, the least stable gene having the largest M value is eliminated first and the M value for resting genes is recalculated until only two genes left. And a lower M value indicates increased stability of candidate reference gene across samples. To screen the optimal combination of reference genes for normalization, the pairwise variation Vn/n+1 between 2 sequential normalization factors (NFn and NFn+1) is calculated, where 0.15 is regarded as the cut-off value. Also, n+1 gene is recommended as the combination of reference genes when Vn/n + 1 is greater than 0.15. On the other hand, however, an additional gene is not required to form the combination of reference genes as Vn/n+1 being lower than 0.15 (Vandesompele et al. 2002). Based on the results of geNorm, the rankings of stability of genes and all of the Vn/n +1 values are output, respectively. To the NormFinder software relying on the mathematical model (https://www.moma.dk/normfinder-software/), the stability value of genes is combined...
by both of the intra- and intergroup variation, and the ranking of stability of genes is completed by depending on the stability value, where the lower stability value, the less systematic error introduced and the more stable gene (Andersen et al. 2004). Subsequently, the overall ranking of stability of genes is calculated by the geometric mean of the above two ranking methods, and we also used the RefFinder software (https://www.heartcure.com.au/reffinder/?type=reference), a web-based comprehensive tool, for further strengthening the analysis (Xie et al. 2012). The optimal combination was screened by the above results.

Validation of reference genes

The findings that the HSP90 was the most stable candidate reference gene and the ACTB was the least one were further confirmed by using the HSP90 and ACTB to normalize the RT-qPCR data of target genes (FASN, HSL, LPL, and PID1) in abdominal and dorsal adipose tissues of Jianzhou da’er goat. Moreover, similar experiments were carried out for the selection of whether a reference gene alone or a combination of reference genes as a suitable reference gene. The RT-qPCR was performed as mentioned above assay, but the concentration of the template cDNA was the 1000-fold dilution of the initial cDNA (namely the $5\times10^{-2}$ ng/μL), and the reaction conditions were set as follows: initial cDNA denaturation at 95°C for 10 min, followed by 39 cycles of denaturation at 95°C for 10 s, 52°C–60°C for 20 s (Table 1), and 72°C for 45 s. The relative expression levels of target genes (FASN, HSL, LPL, and PID1) were calculated by Pfaffi:

$$\text{Ratio} = \frac{(E_{\text{ref}})^{\Delta CT_{\text{ref}} \text{ (control−sample)}}}{(E_{\text{ref}})^{\Delta CT_{\text{ref}} \text{ (control−sample)}}}$$

where E value calculated by the equation $E=10^{-1/\text{slope}}$ indicates the RT-qPCR efficiency of genes transcript, $\Delta CT$ indicates the CT deviation of control-sample of genes transcript, and control means the least CT value (Pfaffi 2001). The significant difference of relative expression levels of target genes (FASN, HSL, LPL, and PID1) in abdominal and dorsal adipose of Jianzhou da’er goat was calculated by the independent-sample T test or the Mann–Whitney U test in SPSS Statistics 17, when the HSP90, ACTB, and a combination of three genes (HSP90, ACTB, and RPLP0) were introduced as the reference genes (Least Significant Difference Test 2008; Tappenden and Miller 2009).

Results

Suitability evaluation of the designed primers

Melt curves analysis of six candidate reference genes were performed to assess their amplification specificity, and all the melt curves have only one single peak, which indicated good specificity of primers with specific products (Figure 1(A–F)). However, the amplification efficiency (98%-105%) and R-square ($R^2 > 0.99$) of six candidate reference genes met the needs of RT-qPCR technique as well (Table 2) (Bustin et al. 2009). On the other hand, the outstanding specificity of primers for four target genes was also identified by their melt curves, but the melt curve of HSL had a double peak, where the value of -d (RFU)/dT of the lower peak was lower than the threshold (Figure 1(G–J)). However, the amplification efficiency and R-square of four target genes also practically satisfied the demands of RT-qPCR technique (Table 2).

Expression profiles of selected genes

The Ct value from the RT-qPCR result is defined as the minimal amplification cycle number, when the fluorescent signal

![Table 1. Information of the primers of candidate reference genes along with target genes.](image)
reaches above the baseline threshold (Zhang et al. 2018). Also, the Ct value is used to represent the mRNA expression level of genes, and the smaller the Ct value is, the more abundantly the gene expressed. For further analysis, we have detected the mRNA expression levels of selected genes in an unbiased using the RT-qPCR technique in our study. As showed in Figure 2(A,B), the histogram of selected genes indicated that the mean values of the Ct value of almost all genes ranged from 20 to 30 with a short error bar showed as the standard error of mean (SEM). On the other hand, the box-plots indicated that the HSP90, in general, was the most stable and expressed

| Table 2. Parameters of RT-qPCR standard curves of six candidate reference genes and four target genes. |
|---------------------------------|-----------------|---------------|--------------|
| Gene          | Amplification efficiency | Slope | R-square |
| ACTB          | 100.9%             | −3.301 | 0.999      |
| ALAS1         | 98.5%              | −3.357 | 1.000      |
| EIF3K         | 103.7%             | −3.237 | 1.000      |
| HSP90         | 98.9%              | −3.348 | 0.999      |
| RPLP0         | 100.2%             | −3.316 | 1.000      |
| UXT           | 104.5%             | −3.220 | 0.997      |
| FASN          | 96.1%              | −3.419 | 0.999      |
| HSL           | 110.6%             | −3.092 | 0.997      |
| LPL           | 93.1%              | −3.500 | 0.999      |
| PID1          | 105.0%             | −3.207 | 0.999      |
gene in these six candidate reference genes in addition to the ACTB and RPLP0 gene possessing the outliers (Figure 2(C)). In contrast, the UXT was the least expressed gene with the highest mean and median of Ct value in the abdominal and dorsal adipose tissue (Figure 2(A and C)). Moreover, as shown in Figure 2(D), almost every Ct value of the target gene was distributed between 20 and 30, and only the PID1 gene had two outliers in the data.

Stability evaluation of candidate reference genes

In geNorm, the average expression stability M values of six candidate genes ranged from 0.887 to 1.059, and the HSP90 and ALAS1 with their M value being 0.887 were identified as the most stable genes in adipose tissues of Jianzhou da’er goat (Figure 3(A)). The UXT was determined to be the third most stable gene, whereas the ACTB having the largest M value 1.059 was the least stable gene in adipose tissues of Jianzhou da’er goat (Figure 3(A)). But based on geNorm analysis, all of the V2/3, V3/4, V4/5, and V5/6 values were higher than 0.15 that is the default limit of 0.15 by geNorm (Figure 3(B)) (Vandesompele et al. 2002). Therefore, we suggested a combination of two genes (HSP90 and ALAS1) as the most stable combination of reference genes in geNorm due to extra reference genes introduced increasing the complexity and instability of the experimental work (Kong et al. 2015). Also, the result of NormFinder showed that high expression stability of six candidate reference genes with the values ranging from 0.361 to 0.615 (Figure 3(C)). The most stable gene was RPLP0 with the stability value being 0.361, whereas the least one was ACTB with a stability value of 0.615 in adipose tissues of Jianzhou da’er goat (Figure 3(C)). Based on the geometric mean of the ranking numbers of geNorm and NormFinder, the overall ranking of stability of six candidate reference genes was HSP90, ALAS1, RPLP0, UXT, EIF3K, and ACTB in adipose tissues of Jianzhou da’er goat (Figure 3(D)). For further verifying the results of the stability evaluation of candidate reference genes, we used the RefFinder software, a web-based comprehensive tool integrated with the geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method (Xie et al. 2012), for ranking the tested candidate reference genes. The ranking of the stability of candidate reference genes was consistent with our previous results (Figure 3(E)), suggesting the previous results were in an unbiased. Considering the above integrated results, we finally accepted that the HSP90, ALAS1, and RPLP0 as a combination was the appropriate reference gene in adipose tissues of Jianzhou da’er goat.

Validation of reference genes

To further validate the suitability of identified reference genes in abdominal and dorsal adipose tissue of Jianzhou da’er goat, the relative expression levels of four target genes (FASN, HSL, LPL, and PID1) were evaluated, when the HSP90 as reference gene compared with the ACTB and a combination of three genes (HSP90, ALAS1, and RPLP0). Clearly, compared with the HSP90 was introduced for normalization, there were always some distinct biases to the relative expression levels of target genes when the ACTB was as reference genes (Figure 4(A–D)), validating the suitability of the HSP90 was better than ACTB. On the other hand, only the transcripts for the HSL and PID1 were changed significantly (P<0.05), when the combination of three genes (HSP90, ALAS1, and RPLP0) was used for normalization compared with that of the HSP90 alone (Figure 4(B and D)). These results proved that the ACTB, in comparison to the HSP90 gene, was the least stable housekeeping gene as the reference, while both the HSP90 alone and the combination of three genes (HSP90, ALAS1, and RPLP0) were accepted to be the suitable reference genes in adipose tissue of Jianzhou da’er goat.

Discussion

In order to improve the meat quality of goats, it is essential to investigate the expression level of adipose-related genes in goat adipose tissue by the RT-qPCR technique (Wood et al. 2004). But the lack of suitable reference genes for the normalization of gene expression in adipose tissue of Jianzhou da’er goat still bothers researchers. The ACTB, a gene encoding actin protein, was one of the most frequently used reference genes for normalization of RT-qPCR data of target genes in human adipose tissue (Gabrielsson et al. 2005). Also, Najafpnah et al. found that the HSP90 was the most stable reference gene in the visceral adipose tissues of the native Iranian goat, while the ALAS1 was the most stable one in the subcutaneous adipose tissue (Najafpnah et al. 2013). In addition, Bonnet et al. suggested that the UXT, EIF3K, and RPLP0 were as the most suitable reference genes in bovine subcutaneous and alpine caprine omental adipose tissues (Bonnet et al. 2013). To Jianzhou da’er goat, however, whether the mentioned six
genes could be used as the reference genes in its adipose tissues remain unclear due to different goat breeds and adipose tissues (abdominal and dorsal adipose tissue).

In the present study, we designed the primers for candidate reference genes (ACTB, ALAS1, EIF3K, HSP90, RPLP0, and UXT) and target genes (FASN, HSL, LPL, and PID1) by Primer Premier 5.0 software. The melt curves and parameters of standard curves of most genes were excellent, except for the HSL, whose melt curve had a double peak. Through analysis, we found that the double peak resulted from the primer dimer, where the fluorescence intensity of primer dimer was beyond that of the target product under the circumstance that an over-low concentration (namely the 5*10^{-8} ng/μL) of template cDNA was used. In contrast, when we used the primers for HSL to detect the mRNA level of HSL in adipose tissues of Jianzhou da’er goat using a 5*10^{-2} of concentration of template cDNA, the relevant melt curve had only one single peak (Information S1 and 2). Given all that, the above problem might result from the primer dimer appearing using a lower concentration of template cDNA than the standard (Ririe et al. 1997). Therefore, the primers for HSL is still acceptable when we used the standard concentration (namely the 5*10^{-2} ng/μL) of template cDNA for the RT-qPCR technique.

The geNorm, NormFinder, and RefFinder softwares were applied to evaluate the stability of candidate reference genes in adipose tissues of Jianzhou da’er goat. The geNorm, a software based on the average expression stability M value to remove the least stable gene until the most stable one output during its work process (Vandesompele et al. 2002), demonstrated that the HSP90 and ALAS1 were expressed as the most stably in adipose tissues of Jianzhou da’er goat, and the least stable candidate reference gene was the ACTB (Figure 2(A)). On the other hand, a single gene was found to be inadequate as a suitable reference gene for normalization of gene expression (Vandesompele et al. 2002; Varshney et al. 2012). Considering additional candidate reference genes added increasing the instability and complexity of experiment, only the HSP90 and ALAS1 were proposed to be a combination of reference genes in geNorm under the condition that all of the Vn/n+1 values were above 0.15 that is a default limit by the geNorm (Figure 2(B)) (Vandesompele et al. 2002; Kong et al. 2015). But for the NormFinder software, the stable...
candidate reference genes were acquired by the principle that the least stability value of genes indicated the best stability, and the \textit{RPLP0} was found to be the most stable reference gene in adipose tissues of Jianzhou da’er goat, while the \textit{ACTB} once more was the least one (Andersen et al. 2004). The different conclusions about the most stable reference genes between geNorm and NormFinder might result from their distinct statistical principle and analytical procedures (Vandesompele et al. 2002; Andersen et al. 2004). The overall ranking of stability of candidate reference genes showed that the \textit{HSP90} was the most stable housekeeping gene followed by the \textit{ALAS1} and \textit{RPLP0} in adipose tissues of Jianzhou da’er goat, which was consistent with Najafpanah et al.’s study to some extent (Figure 2(D)) (Najafpanah et al. 2013). Also, we verified the integrated ranking using the ReFFinder software, whose results were expected by us. Thus, we finally decided to accept the \textit{HSP90}, \textit{ALAS1}, and \textit{RPLP0} as a combination of reference genes in adipose tissues of Jianzhou da’er goat. Also, the ranking of stability of selected reference genes in other researches and ours is showed in Table 3.

For the sake of further validating the suitability of identified reference genes, four lipid metabolism-related genes (\textit{FASN}, \textit{HSL}, \textit{LPL}, and \textit{PID1}) were considered as the target genes to show their relative mRNA expression levels, when we used identified reference genes to normalize the target genes expression. Our [open-strick] [close-strick] results showed that although the \textit{ACTB} was substantiated to be expressed stably in human adipose tissue, it was proved to be the least stable housekeeping gene in adipose tissue of Jianzhou da’er goat (Gabrielsson et al. 2005; Bonnet et al. 2013). Similarly, Jarczak

| Species or Variety       | Adipose tissue                      | Ranking of stability                                      |
|--------------------------|-------------------------------------|----------------------------------------------------------|
| Native Iranian goat      | Visceral                            | HSP90>HMBS>ALAS1>RPS18>GAPDH>ACTB>B2M>ACAC>18s rRNA       |
| Cattle and Alpine goat   | Subcutaneous and omental            | ALAS1>HSP90>HMBS>18s rRNA>ACAC>B2M>GAPDH>ACTB>RPS18       |
| Jianzhou da’er goat      | Abdominal and dorsal                | HSP90>ALAS1>RPLP0>UXT>EIF3K>ACTB                         |

Table 3. The ranking of stability of selected reference genes in different research. (Bonnet et al. 2013; Najafpanah et al. 2013).
et al. also discarded ACTB with the least stable expression as a reference gene in their study, and the expression of ACTB was found to be highly variable in studies involving lipid supplementation of dairy cows as well (Kadegowda et al. 2009; Jarczak et al. 2014). In addition, a combination of reference genes was found to be better than a single one to normalize gene expression (Vandesompele et al. 2002; Varshney et al. 2012). Consistently, in our study, similar transcript patterns (P<0.05) were indicated in the FASN, HSL, LPL, and PIGD, when the HSP90 alone was used as the reference gene for normalization compared with the combination of three genes (HSP90, ALAS1, and RPLP0). In other ways, the HSP90, one of the heat-shock proteins, was suggested to be the most stable reference gene in the liver of the native Iranian goat, and the RPLP0 was regarded as the most stable reference gene in bovine muscle of any breed-related adiposity (Wang et al. 2005; Najafpanah et al. 2013). Also, it was reported that the ALAS1 was the most stable reference gene in prostate tissue, pituitary adenoma, and human retinal endothelial cells under hypoxic and/or hyperglycemic conditions (Ohl et al. 2005; Normann et al. 2016; Xie et al. 2016). The differences among different researchers revealed that the expression of the same housekeeping gene varied considerably in different tissue types and species, and this was why we used a combination of reference genes. Based on these results, we suggested that HSP90 alone and a combination of HSP90, ALAS1, and RPLP0 was the suitable reference genes in adipose tissues of Jianzhou da’er goat.

Conclusion
To our knowledge, this paper was the first to validate whether these six housekeeping genes (ACTB, ALAS1, EIF3K, HSP90, RPLP0, and UXT) could be the suitable reference genes for normalization of gene expression in adipose tissues of Jianzhou da’er goat. A single HSP90 gene and the geometric mean of HSP90, ALAS1, and RPLP0 could be represented as reference genes for normalization of the data from RT-qPCR technique to analyze the relative mRNA levels of genes in adipose tissues of Jianzhou da’er goat.

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