Comparative Study of 13 Candidate Genes Applying Multi-reference Normalization to Detect the Expression of Different Fineness in Skin Tissues of Wool Sheep

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ABSTRACT：This study aimed at finding some novel molecular markers associated with wool traits and providing molecular bases for improved wool quality of sheep. Comparative study of 13 candidate genes was designed to evaluate the different fineness in skin tissue of wool sheep applying 18SrRNA,β-Actin and GAPDH as multi-reference normalization and to detect their expression. The results indicated that significant differences were observed in TXNIP, PIK3CA, TFDP1, ADAM9, FAIM and FZD3 expression in different fineness of wool in the skin tissues of sheep. Comparing the type of wool fineness in sheep, the expression levels of TXNIP, TFDP1 and FAIM genes were higher in the skin tissues of superfine type wool sheep，the expression levels of PIK3CA, ADAM9 and FZD3 genes were higher in the skin tissues of fine type wool sheep. The rest of 7 candidate genes showed no significant difference between the two types.
Keywords: wool sheep; candidate genes; multi-reference; expression

Introduction

Wool fibers are highly organizational structure, scientists believe that tiny polygene effects and control main characters of the wool, and main effect genes may exist controlling this phenomenon (Gat et al. (1998)). Therefore, study of the keratin protein gene family should be a priority while studying the main effect genes controlling wool fiber structure. Adjusting the hair follicle morphogenesis of signaling molecules mainly belong to the family of bone morphogenetic protein, homologous special-shaped box genes, fibroblast growth factor 5 and insulin-like growth factor etc (Yu et al. (2009)).

With the recent advancement of molecular science and technology, candidate genes controlling important economic traits related to fineness and softness of sheep wool cloth have been found. To our knowledge, this was the first comparative study of 13 candidate genes to detect the expression using SYBR Green I method based on multi-reference genes (Tian et al.(2013)). This approach is believed to be useful for selecting novel molecular markers associated with the wool fineness.

Materials and Methods

Skin sample collection. Skin tissues (n=17) from the shoulder of same age wool sheep (9 superfine type and 8 fine type with different fineness of wool) fed on the similar conditions in Xinjiang gong-naisi breeding sheep field were collected. Tissue samples were frozen in liquid nitrogen for RNA extraction and for later use.

Primer design. The primers of both the reference and candidate genes were designed using the conserved sequences of cattle genes in Genbank with Primer 5.0 software and synthesized by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd.

Total RNA extraction and quality control. Total RNA was extracted from skin tissue using a commercially available RNApure Tissue Kit (Tiangen, catalog no. DP431). RNA integrity was assessed in a subset of samples at random using agarose gel electrophoresis. The OD ratio of 28S to 18S was consistently greater than 1 for each sample checked, which indicated high quality RNA.

Two step real-time RT-PCR. Total RNA was converted to cDNA using Quant reverse transcriptase in a reaction volume of 20uL.The reactions were performed according to the manufacturer's instructions with minor modifications. All reactions were performed in triplicate, with non-template controls for each gene.

Data acquisition and Statistical analysis. Applying Roche LightCycler 2.0 fluorescent quantitative PCR system program to calculate amplification efficiency with the formula of amplification efficiency $E=10^{(-1/slope)}-1$, then draw the standard and the melting curves. The expression of candidate genes were normalized by three reference genes (18SrRNA, β-Actin and GAPDH) with the formula $Ct_{(reference)}=\sqrt[3]{Ct_{18SrRNA}\times Ct_{\hat{A}\text{-}Actin}\times Ct_{GAPDH}}$ and calculated by the relative quantification method with $F=2^{-\triangle\triangle Ct}$. Finally, the formula, $R=F_{super\text{-}fine}/F_{fine}$ means the expression ratio between super-fine and fine type. Here Ct means the cycle threshold value; F means the relative quantity of expression. The expression of candidate genes were considered to be significant different in the skin tissues of superfine and fine type wool sheep when $R\geqslant 1.5$.

Results

Amplification efficiency. Based on the slopes of the standard curves and the amplification efficiencies of the standards, all RT-PCR displayed efficiencies between 0.9-1.02. The linear correlation coefficient (R) of all of the selected genes ranged from 0.986–0.991. The Ct of all of the reference and candidate genes in the samples was covered by the range of the standard curves.

Candidate genes expression. Significant differ-

ences were observed in TXNIP, PIK3CA, TFDP1, ADAM9, FAIM and FZD3 expression in different fineness of wool in the skin tissues of sheep. Moreover, the expression ratio between super-fine and fine type was 1.45, 0.61, 1.57, 0.65, 2.55 and 0.52, respectively. Whereas, the 18SrRNA,β-Actin and GAPDH were normalized as reference genes using the established RT-PCR protocol. Totally 13 expressed genes were identified in the superfine type and fine type of fine wool sheep skin tissues in which 3 up-regulated and 3 down-regulated genes were included. Comparing the type of wool fineness in sheep, the expression levels of TXNIP, TFDP1 and FAIM gene were higher in the skin tissues of superfine type wool sheep, the expression levels of PIK3CA, ADAM9 and FZD3 genes were higher in the skin tissues of fine type wool sheep. The rest of 7 candidate genes showed no significant difference between the two types.

Discussion

RT-PCR method for screening candidate genes related to wool fineness. Before quantitative PCR system, the designed primers and the amplification efficiency of primers must ensure its higher sensitivity, the specificity of the PCR amplification and elimination of genomic DNA (Ralf et al. (2006)). This experiment established through optimization and SYBR Green I mode RT-PCR detection system, made in correlation coefficient of standard curve and purpose gene were above 0.99. Amplification efficiencies were from 0.8 to 1.2, which completely accords with the basic standards of quantitative RT-PCR analysis.

This experiment chose 18SrRNA, β-Actin and GAPDH three genes as internal standard, rather traditionally used single internal housekeeping genes. This will not only help deviation correction system to get more reliable results, but also accurate quantitative genes, especially subtle expression differences of molecular research is of significant importance.

Analysis of differentially expressed genes. Tian *et al.*, (2013) has reported that an optimal number of control genes for normalization in skin tissue of fine-wool sheep should be 3 and the most stable genes are 18SrRNA, β-Actin and GAPDH. Therefore, we use multiple reference genes to get more reliable results. All of the 13 candidate genes have been proved to be associated with fiber diameter of fine wool sheep applying expression profile chip (Di et al. (2013)), a similar result was obtained by Di *et al.*, (2013). The chip and RT-PCR results of six genes TXNIP, PIK3CA, PPARD, TFDP1, ADAM9 and FZD3 have similar trends. The correlation analysis indicated that the correlation coefficient between RT-PCR and the expression profile chip is 0.46, which showed the RT-PCR results have good reliability.

The up-regulated genes are mainly involved in the cell nucleus factor NF kappaB cascade control, transcription, lipid metabolism, transmembrane receptor protein tyrosine kinase signaling pathway and other biological processes (Adelson et al. (2004)). The down-regulated genes are mainly involved in protein biosynthesis, phosphorylation, hydrolysis, transport, regulation of cell differentiation, proliferation and Wnt receptor signaling pathways, hydrogen peroxide and oxidizing emergency response and development of biological processes (Jin et al. (2010)). The down-regulated genes involved in the biological process reflect the decrease in wool fineness, its corresponding protein metabolism, cell differentiation and proliferation. Finer the wool is, shorter the fiber and less the wool quantity correspondingly. Thus wool structure requires fewer components accordingly, and it will have good consistency with the biological processes of down-regulated genes (Raufaut et al. (1999)).

Considering the function analysis of differentially expressed genes, most biological processes contains too small number of genes to establish a complete genetic regulatory network for a detailed analysis of its biological function. The reason on the one hand might be relatively small information on hair growth in these resources platform, wheras on the other hand, although there is extensive molecular research on hair biology, which is mainly focused on hair follicles origin and development, and its cyclic growth in human and rat but there is lack of research on regulation of fiber diameter. Differentially expressed genes in this study, however, also identified several biological pathways of the development of hair follicles, and hair growth (WNT, adhesion effect, etc.). It was found that lower gene enrichment in protein synthesis, biological functions such as cell proliferation and apoptosis resulted in reduced fiber diameter and the corresponding length of wool, which showed the trend of decrease in production. The genes of the present study were candidate genes, reported to be candidate genes related to the wool fineness and expressed in the skin.

Wool growth process is relatively complex, and it's not simply the aggregation and expression of keratin, rather the expression is space and time dependent (Rogers et al. (2001)). In addition, gene expression studies need to focus on the signaling pathways, i.e. hair follicle growth related signaling pathways.

Wool diameter is main trait of hair type used in different biological processes. Each follicle progenitor cell number and hair follicle proliferation rate change could change inside hair diameter. Hair diameter change may also be attributed to the cell size or shape of mast cells (such as the flat cells) (Schlake et al. (2004)). At present, in molecular genetics research, the change in hair fiber diameter is rare, and the genetic basis of fiber diameter changes still remains unknown. Studies have shown that knock out and silence Fgfr2 - IIIb gene in mice and transgenic animals caused by the decrease of the medulla layer of the hair shaft refinement can be attributed to influence cell proliferation. Mouse follicle size change seems to be consistent with the hair length and diameter change. Some wild type hair (such as Guard hairs, awl dovetail) length and diameter changes are related to the hair follicle size, the most common reason is due to the medulla layer change. Different from the above hair type, fine wool and cashmere fiber structure does not contain medulla layer. Moreover, the influence of cell proliferation, cell size and shape on fiber diameter can be used in future research for

wool improvement in fine wool type sheep.

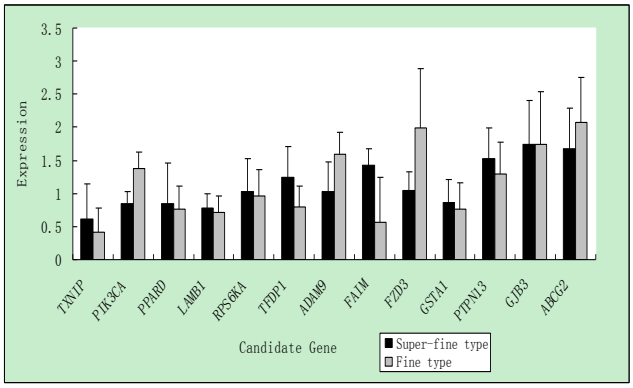

Figure 1 The expression of candidate genes in skin of sheep with super-fine and fine wool

Conclusion

In the present research,18SrRNA, β-Actin and GAPDH three genes were selected as multiple reference genes to get more reliable results. Significant differences of TXNIP, PIK3CA, TFDP1, ADAM9, FAIM and FZD3 genes expression were observed in different fineness of wool in the skin tissues of sheep. This could provide a foundation for molecular breeding and improvement of the wool traits in sheep.

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