Cloning, Sequence Analysis, and Tissue Expression of the KITL Gene in Goat (Capra hircus)

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ABSTRACT

Kit-ligand (KITL) plays a crucial role in skin melanocyte development and maintenance. However, the KITL function and its influence on Youzhou dark goat skin pigmentation remains unclear. The present study cloned goat KITL gene sequence, predicted the physicochemical properties of the gene-encoding protein, and analyzed the different expression level in goat tissue. The result showed that the coding region of goat KITL gene was 825-bp long and encoded 274 amino acids. The goat KITL amino acid sequence had high homology with this molecule in other mammalian species. And the KITL gene was extensively expressed at 0-, 12-, and 24-month-old goat tissue. Moreover, the mRNA and protein of KITL were increasing at 0-, 12-, and 24-month-old goat skin, which were confirmed by real-time qPCR, western blotting and immunohistochemical analysis. This study provides a theoretical basis for further clarifying the biological function of the KITL gene in goat.

INTRODUCTION

Melanin is a biological polymer that widely exists in plants, animals and microorganisms. Animal hair, skin, and eye color are mainly determined by the quantity, type, synthesis, and distribution of eumelanin and pheomelanin, which are produced by melanocytes (Cesarini and Nserm, 1996; Ito et al., 2000). Melanocytes are responsible for both synthesizing melanin in melanosomes and transporting melanosomes to the surrounding epidermal cells (keratinocytes) (Cesarini and Nserm, 1996). The main extracellular signaling pathways for melanin production are ASIP/α-MSH/MC1R, SCF/c-kit, Wnt/β-catenin and ET-1/PKC, which regulate melanocyte biological characteristics and melanin synthesis. To date, numerous genes are known to be involved in melanocyte development, migration, formation, and pigment deposition (Emfors, 2010). Melanin synthesis is orchestrated by the coding products of multiple gene loci, which suggests that pigment synthesis is controlled by a complex regulatory network.

Kit ligand (KITL, also called stem cell factor, mast cell growth factor, or steel factor) plays a crucial role in the development and maintenance of the melanocyte lineage. KITL exerts survival, proliferation, and migration functions in KIT receptor-expressing melanocytes (Wehrle-Haller, 2010). Moreover, decrease of KIT receptor tyrosine kinase signals could lead to irregular melanocyte development. Additionally, mice with the KITL gene mutation have complete absence of pigmentation and male infertility (Aoki et al., 2009). Moreover, melanocytes in the eyes and ears are less sensitive to KIT signals than those in the skin (Wehrle-Haller and Weston, 1999). KITL both supports melanocyte survival and proliferation, and promotes cell differentiation (Wehrle-Haller, 2010). As a transcriptional target of p53, KITL-mediated up-regulation affects skin pigmentation (Pant et al., 2016). Moreover, the stem cell factor/receptor tyrosine kinase (SCF/c-kit) signaling pathway is essential for melanocyte survival, proliferation, and differentiation. When SCF binds to the membrane receptor c-kit, the c-kit receptor phosphorylates and activates multiple signaling pathways to regulate melanin synthesis (Yamada et al., 2013). Youzhou dark goat, which has a natural skin color mutation, has dark skin on its body, including visible mucous membranes, but a generally white coat color (Ren et al., 2017). Therefore, the aim of this study was to investigate KITL gene cloning, sequence analysis, and tissue expression in Youzhou dark goat.

In this study, the KITL gene sequence was cloned in Youzhou dark goat skin, and the KITL amino acid sequence was further assessed by bioinformatics analysis. The KITL mRNA expression profile was detected in 0-, 12, and 24 months old Youzhou dark goat tissues by real-time qPCR. Furthermore, the protein level and localization of KITL expression were further examined by Western
blotting and immunohistochemistry at three growth stages (0, 12, and 24 months) of goat skin. This study both provides a theoretical basis for further clarification of the biological function of the \textit{KITL} gene in goat and also uses the Youzhou dark goat as a model to provide a novel perspective for ongoing genetic and molecular studies on skin pigmentation.

**MATERIALS AND METHODS**

**Animals and sample collection**

All surgical procedures involving goats were approved by the Animal Care and Use Committee, Chongqing, People’s Republic of China. Tissue samples including heart, liver, spleen, lung, kidney, longissimus dorsi, and ventral skin were from purebred Youzhou dark goat in Youyang County, Chongqing, China (26°54′ N, 108°57′ E). Three pregnant goats were subjected to caesarean section to collect the ram fetuses at 100 days of gestation (recorded as 0 months of age). Tissue at 12 and 24 months of age (three ram samples in each age group) were slaughtered, dissected, and quickly stored in liquid nitrogen.

**RNA extraction and cDNA synthesis**

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer’s instructions. RNA purity was detected using a spectrometer (Nanodrop, USA) at ratios of OD 260/280 between 1.8 and 2.1. RNA integrity was assessed using agarose gel electrophoresis. The Transcriptor First Strand cDNA Synthesis Kit (Roche, Germany) was used, and 1 µg of RNA was transcribed into cDNA. The cDNA was then stored at −20°C for further analysis.

**PCR amplification, cloning and sequence analysis**

The primers were designed based on the gene sequences of the common goat \textit{KITL} (\textit{Capra hircus}, NM_001285670) in the NCBI database (https://www.ncbi.nlm.nih.gov/). The goat skin cDNA was amplified by PCR. The PCR procedure was as follows: 94°C for 3 min; 34 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 55 s; 72°C at 5 min; and then 4°C for storage. PCR products were electrophoresed, recovered, and purified if they were the same length as the target band. The purified correctly sized PCR products were cloned into pEASY-T1 Cloning Vector (TransGen Biotech, China) and then converted into a Trans5α-competent cell. Positive clones were randomly selected and then sequenced by Shanghai Sangon Biotech (China).

The sequencing results were analyzed by ORF finder and BLASTN in the NCBI website to identify gene open-reading frames (ORFs) and sequence homology. The online software programs ExPaSy, ProtScale, SWISS-MODEL, and DNAMAN were used to analyze the physicochemical properties, and secondary and tertiary structure of proteins.

**Quantitative real-time PCR**

Gene expression was analyzed using FastStart Universal SYBR Green Green Master (ROX) (Roche, Germany). The qRT-PCRs were performed using 1 µL cDNA, 0.5 µL forward primer (30 µM), 0.5 µL reverse primer (30 µM), 6 µL FastStart Universal SYBR Green Master (ROX), and 2 µL ddH2O in a 10-µL volume. The qRT-PCR conditions were as follows: 10 min at 95°C followed by 40 cycles for 15 s at 95°C and 60 s at 60°C. The mRNA transcripts were detected using QuantStudio (TM) 6 Flex System (Applied Biosystems, USA). Gene expression was calculated using the 2−ΔΔCt method. The expression data were normalized to the expression of a house-keeping gene (ACTB), and the primer information was listed in Table I.

**Western blot**

Total protein was extracted from skin with RIPA lysis buffer supplemented with protease inhibitor. Proteins in lysates were resolved by SDS–PAGE, transferred to a PVDF membrane, blocked with 5% BSA, and then incubated with primary antibodies against \textit{KITL} (1:800, Proteintech, China), and ACTB (1:1000, Bioss, China). Membranes washed (3×5 min) with TBST proteins were visualized using horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG-HRP, 1:1000, Bioss, China) and were analyzed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo, USA). The membranes were assessed using a GS-800 calibrated imaging densitometer (Bio-Rad, USA) and calculated using Image J software.

**Immunohistochemistry**

Skin tissues were fixed in 10% formalin in phosphate-buffered saline (Solarbio, China) for 15 days and embedded in paraffin. The paraffin sections were dewaxed and hydrated, and then boiled in the antigen repair solution (0.01 mol/L sodium citrate buffer, pH 6.0, Solarbio, China). The sections were blocked using normal rabbit serum (Bioss, China) after \textit{KITL} antibodies (1:400, Proteintech, China) were added. The sections were allowed to sit overnight at 4°C, washed three times (5 min each) with phosphate buffer solution (PBS, Solarbio, China), and incubated with HRP-conjugated anti-rabbit IgG secondary anti-bodies (Bioss, China). Immunoreactivity sites were visualized using a bright-field microscope (Olympus Dp71, Japan).
Table I. Primer information of the goat KITL gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
<th>Product size, (bp)</th>
<th>Application</th>
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</table>
| KITL-CDs | F: ATGAAGAAGACAAACAACTGGATTATCAC  
                     R: TTACACTTCTGAAACCTCTCTCTTTTC | 825bp            | cloning         |
| KITL   | F: CCTCCAATGCTATCAGGGT  
                     R: GTCAATGCAAAACAAACAG | 134bp            | RT-qPCR         |
| ACTB   | F: ATGCGAGACAGGATGCAGAA  
                     R: CATCTGCTGGAAGGTTGACAA | 141bp            | RT-qPCR         |

Statistical analysis
Each experiment was independently performed in triplicate. Results are shown as the mean ± SD.

RESULTS

Clone of the goat KITL gene

RNA extracted from Youzhou dark goat skin was synthesized by cDNA reverse transcription and detected by agarose gel electrophoresis. The molecular weight of the specific amplification product was 825 bp and positive and negative theory between primer fragment length (Fig. 1a). Because it matched the expected fragment size, subsequent cloning was performed. The consistency reached 99% after comparing the cloned sequence with previously published sequence identified by blastn in NCBI (Fig. 1b). This finding indicated that the sequence amplified by KITL-CDs primer were goat KITL gene sequence.

Amino acid sequence analysis of the goat KITL gene

The PCR product sequencing result showed that the goat ORF initiation codon of the KITL gene was ATG, the termination codon was TAA, and this gene encoded 274 amino acids (Fig. 1b, 2a). Alignment of the predicted protein with homologous protein from various species in DNAMAN indicated that the goat KITL amino acid sequence had high homology with this molecule in other mammalian species, the phylogenetic tree showed that the goat KITL gene was most closely related to that of sheep (Figs. 2a, 2b). Besides, the molecular weight, molecular mass, and amino acid composition were analyzed by the ExPASy online tool. The molecular weight of the goat KITL protein was 31 kD and had a molecular formula of C\textsubscript{1380}H\textsubscript{2204}N\textsubscript{360}O\textsubscript{425}S\textsubscript{13}. With regard to amino acid composition, the Ser content was as high as 12.4%, whereas Pyl and Sec contents were the lowest (0.0%). The total number of negatively charged residues (Asp+Glu) was greater than that of positively charged residues (Arg+Lys) (Fig. 2c). The KITL protein had secondary and tertiary structure junction were shown in Figure 2d, 2e, and consists of eight alpha helices and three beta folds in the order of ααβααααβαα.

The mRNA expression profile of KITL gene in goat tissues

To examine the spatiotemporal and tissue expression specificity of KITL, the KITL gene expression in different tissues of 0-, 12-, and 24 month old Youzhou dark goat by real-time qPCR. Result indicated that the KITL gene was extensively expressed in 0-, 12-, and 24 months old goat tissue. The KITL gene was abundantly expressed in...
skin, heart, spleen, lung, kidney, and muscle, but weakly expressed in liver. Besides, the \textit{KITL} gene was increasing in 0-, 12-, and 24 months old goat skin (Fig. 3).

KITL in goat, the KITL protein expression in Youzhou dark goat skin by western blotting. The KITL protein was highly detected in 0-, 12-, and 24 months old goat skin (Fig. 4a). Protein relative abundance was presented as the ratio of the intensity of the protein to that of ACTB. The relative abundance of the KITL protein increased over time in 0-, 12-, and 24 months old goat (Fig. 4b).

Fig. 2. Amino acid sequence analysis of the Youzhou dark goat \textit{KITL} gene. a, homology of goat, sheep, bovine, rabbit, pig, mouse, and human KITL amino acid sequence. Sequence was aligned in DNAMAN. Amino acid sequence homology is shown by black shading (100%), pink shading (≥75%), and blue shading (≥50%). b, phylogenetic tree of KITL amino acid sequences. c, amino acid composition of goat KITL as determined in EXpASy. d, secondary structure prediction analysis of KITL conducted by Jpred. H (red): α-helix; E (yellow): β-sheet. e, tertiary structure of the KITL protein in goat, sheep, bovine, rabbit, pig, mouse, and human.

\textbf{KITL protein expression and localization}

To further determine the protein expression level of KITL in goat, the KITL protein expression in Youzhou dark goat skin by western blotting. The KITL protein was highly detected in 0-, 12-, and 24 months old goat skin (Fig. 4a). Protein relative abundance was presented as the ratio of the intensity of the protein to that of ACTB. The relative abundance of the KITL protein increased over time in 0-, 12-, and 24 months old goat (Fig. 4b).
Then localization of KITL expression during those developmental stages in goat skin were confirmed. Immunohistochemistry was performed to assess the abundance pattern of this protein in goat skin. The KITL was expressed in 0-, 12-, and 24 months old goat skin (Fig. 4c). The immunohistochemical results were very consistent with those of western blotting.

**DISCUSSION**

Youzhou dark goat, which characterize the dark skin of whole body including the visible mucous membranes (Ren et al., 2017). In this study, the KITL gene was cloned from goat skin. The result suggested that the coding sequence of the goat KITL gene was 825-bp long and encoded 274 amino acids, the molecular weight of the goat KITL protein was 31 kD and had a molecular formula of C_{1238}H_{2204}N_{300}O_{380}S_{13}. This first confirmed some detailed information on the goat KITL gene. The goat KITL amino acid sequence had high homology with this molecule in other mammalian species and the goat KITL gene was most closely related to that of sheep. This was mostly consistent with the previous findings of the KITL gene sequence analysis between fish, salamanders, frogs, birds, and mammals (Huang et al., 1992; Parichy et al., 1999a, b), which may suggest that the biological function of KITL in vivo is quite stable, and their roles in the development of melanocyte production are relatively conserved. Meanwhile, the Ser content reached 12.4%. Ser is one of the 20 common amino acids, a non-essential amino acid in mammals, and a ketogenic amino acid. Ser boosts fat and fatty acid metabolism and helps maintain the immune system. The high Ser content in the KITL protein may also be related to pigmentation and the abnormal metabolic process of producing melanin that is expressed in goat skin. However, the detailed regulatory mechanism still needs to be further studied. Abundant alpha spirals help maintain protein stability (Kumar et al., 2000; Querol et al., 1996). The KITL protein had the following secondary structure ααββααααββαααααααα. The proportion of alpha helices is low, which leads to its poor stability. The stability of the goat KITL protein may also be related to its physiological function.

KITL, also called stem cell factor, is a growth factor that exists both as a membrane-bound and soluble form with highly conserved cytoplasmic domains. It is essential for proliferation, migration, survival, and differentiation of hematopoietic progenitors, melanocytes, germ cells, and mastocytes (Pauhe et al., 2004), which indicates that KITL plays a key role in development of different tumor cells. KITL is expressed in the branchial arch mesenchyme of the head and in the dorsal aspect of the dermatome in the trunk and tail (Wehrle-Haller and Weston, 1995). The adult mouse epidermis does not express KITL nor melanocytes, and melanocytes are localized to the hair bulb (Nishimura et al., 1999). Moreover, KITL is expressed in the adult human epidermis, where it plays an important role in the homeostasis of epidermal, Kit receptor-expressing melanocytes (Grichnik et al., 1998; Spritz et al., 1994). The findings of the previous studies indicated that KITL expression is involved in melanocyte development.

In our study, the KITL gene was extensively expressed in 0-, 12-, and 24 months old goat tissue, and the KITL gene was abundantly expressed in different tissues. Besides, the KITL gene was increasing in 0-, 12-, and 24 months old goat skin. These real-time qPCR results differed from those of adult mouse epidermis, which does not express KITL nor melanocytes (Nishimura et al., 1999). And the mice without a KITL gene had complete absence of pigmentation and male infertility (Aoki et al., 2009). Perhaps the Youzhou dark goat itself has a natural skin color mutation, the mouse is only normal or factitious organism. This reflects the complexity of gene expression in different species. In this study, the relative mRNA expression profile of the KITL gene in lung and kidney were higher than that of skin, and the expression profile in liver was lowest. This suggest KITL gene may be involved in viscera development, and it also could play a role in goat adaptation, immune, and metabolism aspects. Importantly, the protein level and localization of KITL were further expressed in 0-, 12-, and 24 months old goat skin. These findings further confirm that the KITL gene was expressed in different developmental phases of goat, which indicates that the KITL gene might be relevant to skin pigmentation. KITL promote melanocyte survival and proliferation, and promotes cell differentiation (Wehrle-Haller, 2010) and KITL gene is involved in skin pigmentation (Pant et al., 2016). Gene expression in animal tissues is influenced by genetic factors, nutrition level, environment, wildlife management, and disease. However, there are few studies on KITL gene expression in economic animals, and these results need to be further verified. To comprehensively understand how KITL is associated with pigmentation, the roles that this gene plays in migration, proliferation, or differentiation of melanocytes, and its regulatory role in the skin pigment process need to be elucidated. Such information would enhance our understanding of the molecular mechanisms underlying KITL's involvement in skin pigmentation regulation and provide valuable information that can be used to help elucidate human melanocytosis.
CONCLUSION

In summary, the coding region of goat KITL gene was 825-bp long and encoded 274 amino acids, the goat KITL amino acid sequence had high homology with this molecule in other mammalian species. The KITL gene was extensively expressed at 0-, 12-, and 24 month old goat tissue. Moreover, the mRNA and protein of KITL were increasing at 0-, 12-, and 24 month old goat skin, which were confirmed by real time qPCR, western blotting and immunohistochemical analysis. This study provides a theoretical basis to help elucidate the biological function of the KITL gene in goat.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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