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Immunohistochemical distribution of c-Kit and its gene expression via real-time PCR in skin tissue of goats with a black and white coat colour*

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The signaling pathway of the stem cell factor and its receptor, c-Kit, plays an important physiological role in melanogenesis. The expression of c-Kit was investigated using real-time quantitative PCR and immunohistochemical staining in skin with a white and black coat colour. The length of the entire open reading frame of the *c-Kit* gene is 2925 bp. The c-Kit protein is conserved in the structure domain. The c-Kit is mainly located on membranes and widely expressed in the skin tissue. The mRNA expression of *c-Kit* is 1.28 times higher in skin with the black coat colour than in that with the white coat colour. There was no significantly different expression of *c-Kit* mRNA and protein ($P>0.05$) between skin with the white and the black coat colour. These results indicate that there seems to be no direct relationship between coat colour and the expression of the *c-Kit* gene in goats.

KEY WORDS: c-Kit gene / coat colour / expression / goat / skin tissue

Colour variation of feathers, fur and skin is largely determined by the types and ratio of melanin in melanocytes [Lin and Fisher 2007]. Hair colour and markings in mammals depend on the type and amount of melanin present in the hair shaft. The

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balance between brown/black eumelanin and red/yellow pheomelanin is a key factor in mammalian hair/coat colour. Melanocytes are located in the epithelial cell matrix of the anagen hair bulb. Melanin is synthesised in melanocytes and exported to adjacent keratinocytes in the bulb via melanosomes [Randall *et al*. 2008].

Mammalian hair/coat colour is regulated by multiple *loci*. The mast/stem cell growth factor (SCF) is encoded by the *steel* (*sl*) locus, while c-Kit is encoded by the *dominant white spotting* (*W*) *locus* in the mouse [Bernstein *et al*. 1990, Tan *et al*. 1990]. The *c-Kit* gene encodes the receptor for the SCF, or Kit ligand (Kitl). The c-Kit protein with the calculated molecular weight of 109.8 kDa has three main types of structure: an extracellular domain consisting of five Ig-like domains, a transmembrane region and a tyrosine kinase domain [Fontanesi *et al.* 2010]. SCF/c-Kit signaling is required for the maintenance of melanocytes in hair follicles and plays a pivotal role in regulating pigmentation in mammalian hair [Botchkareva *et al*. 2001, Hachiya *et al.* 2009]. Mutations in either *Kitl* or *c-Kit* in mice result in the absence of melanocyte populations in the skin and inner ear, sterility, anemia, mast cell deficiencies and in some cases, stillbirths [Bernstein *et al.* 1991, Williams *et al.* 1992, Galli *et al.* 1994]. Mutations in the *c-Kit* gene cause piebaldism, where patients develop amelanotic patches on ventral and/or acral skin surfaces [Giebel and Spritz 1991]. The variations in pig coat colour are known to be determined by different alleles of *c-Kit* [Giuffra *et al.* 1999, Welker *et al.* 2000, Hermes *et al*. 2001, Pielberg *et al*. 2002, Lan *et al.* 2008]. Different pigmentation patterns and colours in the horse are derived from mutations in the *c-Kit* gene [Kunisada *et al*. 1998, Brooks and Bailey 2005, Haase *et al*. 2007]. The *c-Kit* gene is expressed in cultured follicular melanocytes and hair bulbs, where pigment is formed [Randall *et al*. 2008]. Previous reports focused on the association between mutations of the *c-Kit* gene and coat colour distinction in several species. To our knowledge no relationships between the expression level of c-Kit in skin and coat colour in goat have been identified. The Taihang goat is a Chinese indigenous breed with a distinctly different coat colour. The aim of this study was to investigate the *c-Kit* gene in skin tissue, to determine the c-Kit structure and the relationship between the expression level of the gene and coat colour in the skin of Taihang goats.

Material and methods

Collection of skin biopsies

In total, twelve goats were selected, six with the white coat colour and six with the black coat colour. The skin biopsies were collected, snap frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. The skin biopsies collected from the same animal were formaldehyde-fixed and paraffin-embedded for immunohistochemical analyses.

RNA isolation

Total RNA was isolated from the stored skin biopsies with an EasyPure® RNA Kit according to the manufacturer's protocol (TransGen Biotech, Beijing, China).

The RNA concentration was measured using the Nanodrop 2000 instrument (Thermo Scientific). The nucleic acid concentration was diluted to10 ng/µl. First-strand cDNA was synthesised from the total RNA, using MMLV reverse transcriptase and an oligo $d(T)_{18}$ primer and stored at -20°C. Reverse transcription (RT) was carried out from 1.2-1.5 ng RNA in a total volume of 50 µl, containing 5 pmol oligo $d(T)_{18}$ (Takara, Dalian, China), 0.5 mM deoxyribonucleoside triphosphate (dNTPs), 1×RT buffer, 1 U of RNase inhibitor (Takara, Dalian, China) and 5 U reverse transcriptase (Takara, Dalian, China) according to the manufacturer's instructions. The reaction mixture was incubated for 60 minutes at 42°C and then heated for 15 minutes at 70°C and next cooled on ice.

Polymerase chain reaction

The first-strand cDNA was used for PCR amplification. The reactions were performed in a volume of 50 µl containing 1 x ES Taq MasterMix (CWBIO, Beijing, China), 0.4 µM specific primers and 20-25 ng/µl cDNA. Amplification conditions were as follows: denaturation at 94°C for five minutes, followed by 36 cycles of 94°C for 30 seconds, 62°C (annealing temperature) for 30 seconds, and 72°C for one minute with a final extension at 72°C for 10 minutes. Specific primers for the amplification of c-Kit in skin were designed based on the homology between the goat (GenBank Acc. no. D45168) and cattle (GenBank Acc. no. NM_001166484).

Cloning and sequencing

All of the amplicons were purified using the Gel Extraction Kit (Sangon Biotech, Shanghai, China). Cloning was performed in the TA cloning system (pEASY-T3, TransGen Biotech, Beijing, China). The ligated products (5 µl) were transformed by the heat shock treatment into a competent cell, trans1-T1 (TransGen Biotech, Beijing, China). Positive colonies were identified using PCR and restriction enzyme digestion according to the vector map. The plasmids were isolated with the plasmid miniprep kit (Sangon Biotech, Shanghai, China) and sequenced with M13 forward and reverse primers by the Beijing Genomics Institute (Beijing, China). Sequences were viewed with the Chromas program. The sequences were assembled with the Seqman program in the DNASTAR package.

Real-time quantitative PCR

The primers (Tab. 1) for *c-Kit* were designed from the goat c-Kit sequence (GenBank Acc. no. D45168) using the Primer Premier (version 5.0) software. The *GAPDH* gene was used as an internal reference gene. Real-time quantitative PCR was performed in triplicate on 1µL cDNA from six white or black goats using SYBR qPCR Mix (2×) (Toyoba Life Science, Shanghai, China). Amplification was performed using the Bio-Rad iQ5 PCR system (Bio-Rad Laboratories, Inc.) under the following cycling conditions: denaturation at 95°C for 15 seconds, 40 cycles at 95°Cfor 15 seconds, 62°C for 15 seconds and 72°C for 45 seconds. The amplification mixture without a template served as a blank control. Melting temperature curve analysis was performed

following PCR to determine the specificity of PCR products. The housekeeping gene, *GAPDH,* was amplified in parallel with the *c-Kit* gene and the relative gene expression level was calculated by the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers for amplification of c-kit and Real-time PCR

Target	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Product size (bp)
Exon1-5	CTAGAGCCGGAACGTGGA	GCCTTTCCTGACGGAGATAACT	55	875
Exon4-10	AGCCAGCAGAGTAAAGCACA	TCACAAAACCAATCAGCAAC	54	844
Exon10-18	CCATGCTCACACCCTGTTC	GGTGCCATCCACTTCACAG	53	969
Exon18-21	ACTCCCTGTGAAGTGGATGG	TGGGTGATTATGAGGAATGAAG	55	800
c-kit qPCR	GGCCCACCCTGGTCATTACAGAATAT	TGTCTGCCGCCTTGGTTGGTAC	62	225
GAPDH qPCR	CACCCTCAAGATTGTCAGC	CAGTGGTCATAAGTCCCTCC	62	107

Immunohistochemical staining

Special care was needed during sectioning to orientate the specimen in order to obtain longitudinal sections of hair follicles. Vertical formalin-fixed skin sections (5-8 mm) were cut and collected on APES-coated slides. APES (Zhongshanjinqiao, Beijing, China) diluted 1:50 with acetone was used to clean the slides. All staining was done in a moistened chamber at room temperature.

Sections were dried at 57°C for 24 hours. Sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Endogenous peroxidase activity was blocked by treatment with a 3% hydrogen peroxide:methanol (1:4) solution for 20 minutes. Non-specific binding sites were blocked by incubation with goat serum (CWBIO, Beijing, China) for 20 minutes, followed by incubation with a primary mouse anti-rabbit monoclonal antibody (1:80) (Santa Cruz Biotechnology, USA) for 16 hours at 4°C. A negative control of parallel incubation with PBS solution was carried out. The sections were washed three times with PBS solution (5 minutes for each) and incubated with biotinylated anti-rabbit immunoglobulins (CWBIO, Beijing, China) for 30 minutes. The sections were incubated with four to six drops of Streptavidin-HRP for 20 minutes. Similar levels of DAB staining were followed by the next wash for about 3-5 minutes; brown colour appearance was microscopically monitored. Sections were restained with hematoxylin, dehydrated with an ethanol gradient series, cleared in xylene and fixed with neutral balata.

Sequence analysis and molecular phylogeny

The sequence was edited and translated using the BioEdit and DNASTAR program. The position of exons and introns was determined using WebScipio (http://www.webscipio.org/) in reference to the c-Kit gene structure of cattle. Multiple alignment of sequences were performed with ClustalW. Bioinformatic analyses were performed with online programs on NCBI (http://www.ncbi.nlm.nih.gov/) and Expasy (http://www.expasy.org/tools). An *in silico* comparison of genomic sequences was performed on the nucleotide sequence of the *c-Kit* gene from the Ensembl and NCBI

database in five species: the human (GRCh37:4:55524085:55606881:1), mouse (GR Cm38:5:75574916:75656722:1), dog (CanFam3.1:13:47107802:47192057:1), cattle (UMD3.1:6:71796318:71917431:1) and goat (GenBank Acc. No. AJPT01069460). The nucleotide sequences of the five species were aligned using the mVISTA program (http://genome.lbl.gov/vista/index.shtml). The VISTA plot displayed a 50-100% sequence identity between cattle and the other species.

Results and discussion

Cloning of goat skin c-Kit and sequence analysis

The full-length coding sequence of the *c-Kit* gene was obtained using RT-PCR by overlapping the four fragments that were amplified using the primers shown in Table 1. The sequence length was 2925 bp, which encodes a protein of 974 amino acid residues, with a predicted molecular weight of 109722 Da, and which theoretical isoelectric point is 6.32. The signal peptide was identified from the 1st to 25th amino acids at the N-terminus, according to the SMART analysis (http://smart.embl-heidelberg.de/). There are three immunoglobulin (Ig) domains, two Ig-like structures as well as one transmembrane region and tyrosine kinase catalytic domain. The putative topology of goat c-Kit contains one transmembrane domain from amino acids 518-540 near the C-terminus according to the Prosite and the TMHMM programs. Two splice variants were found through BLAST analysis. The length of the entire coding sequence was 2937 bp (GenBank Acc. No. D45168), which encodes a protein of 978 amino acid residues due to the insertion of four amino acid residues (GNNK) at position 511. The genomic alignment of the *c-Kit* gene between goats and the other four species showed sequence identity of exons are greater than 75% (Fig. 1).

Chromosome location and genomic structure of the caprine *c-Kit* gene

Upon scanning through the goat genome CHIR_1.0 (2013/1/11) we attained a sequence size of 80.4 kb (GenBank Acc. no. AJPT01069460) on chromosome 6. This represents approximately 66% of the known *c-Kit* gene size when compared to cattle (121.114kb) (source: Ensembl). The gene encoding the caprine c-Kit is located within a syntenic group on chromosome 6. The portion of caprine chr6 is homologous to chr6 in cattle (Btau 4.6.1 primary assembly, NW_001495197.4) or sheep (Oar v3.1, NC019463). Comparison and prediction of genomic DNA and cDNA sequences reveals that the caprine *c-Kit* gene consists of 21 exons interrupted by 20 introns when compared to the gene structure in cattle (Fig. 2).

Expression of goat c-Kit in skin tissue with black and white coat colour

To examine variants at the transcriptional level, total RNA was isolated from the caprine skins with white and black coats. The relative mRNA expression level was measured by Real-time qPCR and calculated with the $2^{-\Delta\Delta Ct}$ method, where the ΔCt is the "c-Kit Ct-GADPH Ct." The expression of c-Kit mRNA is 1.28 times higher in skin

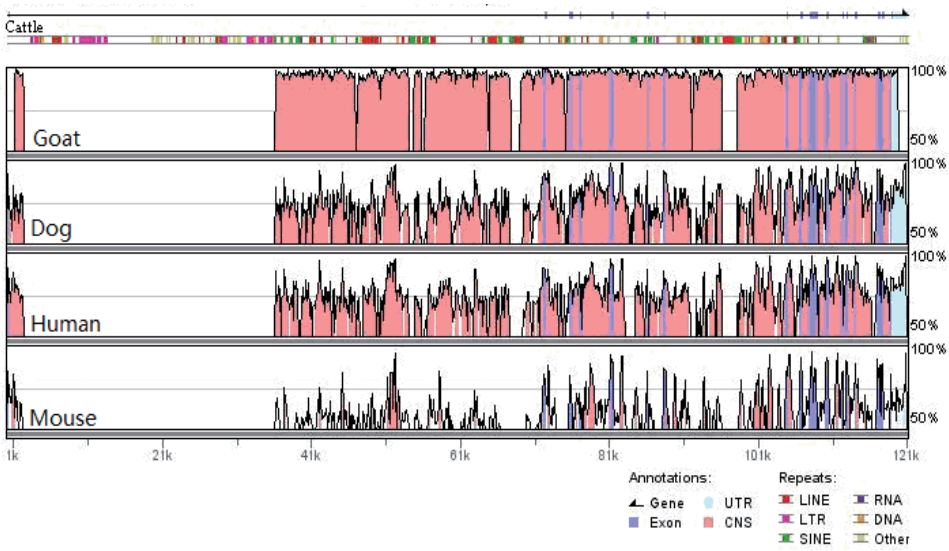

Fig. 1. Nucleotide sequence identity of *c-Kit* gene between cattle and other species. Peaks show their per cent identities (50-100%, vertical axis) at their relative positions to the reference cattle sequence (horizontal axis).

Fig. 2. Gene architecture of caprine c-kit gene in reference to cattle.

with the black coat colour than in that with the white coat colour (Fig. 3). There was no significant difference in c-Kit expression between skin of black and white coloured goats ($P>0.05$).

Immunohistochemical localisation of c-Kit in skin samples

The distribution of c-Kit in caprine skin tissue with black and white coat colour is shown in Figure 4. Large amounts of visible melanin pigment can be seen in goat hair. The c-Kit is mainly located on the membrane, as well as the epithelium, follicular infundibulum, outer and inner root sheaths, sebaceous and sweat glands in goat skin tissue. The c-Kit staining was negative in the control group with no primary antibody incubation. The results of mean positive optical density analysis showed that there was no significant difference in c-Kit staining between skin tissues from black and white coat coloured goats.

We reported the complete coding sequence of the c-Kit gene and for the first time analyzed the relationship between the expression level of goat *c-Kit* in skin and the coat phenotype. The predicted kit protein encoded by the goat *c-Kit* gene

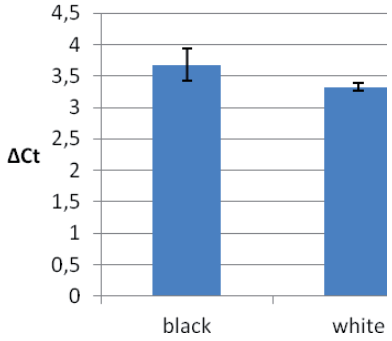

Fig. 3. Comparison of the *c-Kit* gene expression as measured by real-time qRT-PCR. The average expression in the skins from each of six goats is shown as meanΔCt values±SEM.

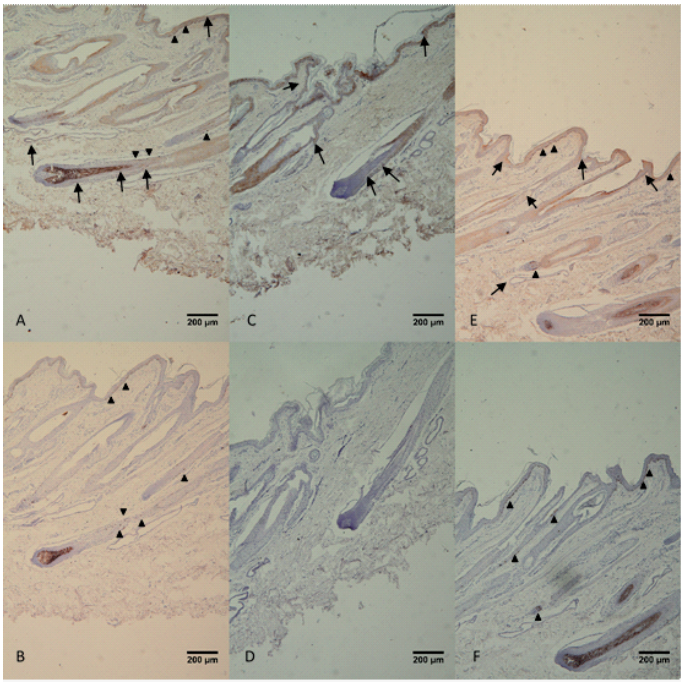

Fig. 4 Distribution of c-KIT in goat skin tissue by IHC (536 ×). c-Kit in the inner and outer root sheath of hair follicles, sebaceous glands and sweat glands in black goat skin tissue (A) and control (B); c-Kit in the inner and outer root sheath of hair follicles, sebaceous glands and sweat glands in white goat skin tissue (C) and control (D); c-Kit in skin epidermis and infundibulum in black goat skin tissue (E) and control (F) ➜ showing c-Kit positive parts; ▲ showing melanin.

has a conservative structure domain shared with other species. The comparison of the genomic sequences of *c-Kit* between the goat and other species revealed that *c-Kit* is relatively conserved between five species. In the phylogenetic analysis

the genetic distance is smallest between *Capra hircus* and *Bos primigenius*, *Bos taurus* or *Bubalus bubalis*. The caprine kit receptor contains 974 amino acids with a signal peptide, extracellular domain, transmembrane region and a tyrosine kinase domain. The predicted structure is consistent with the reported human protein. The human c-Kit is expressed as a 145-kD glycosylated transmembrane protein with an extracellular domain, a transmembrane region and a tyrosine kinase domain. The extracellular domain consists of five Ig-like domains. The first three Ig-like domains of the membrane-bound kit (m-Kit) are involved in binding SCF, while the fourth Ig-like domain of this kit is involved in receptor dimerisation. Binding and dimerisation of Kit subsequently causes autophosphorylation at tyrosine residues, followed by the activation of downstream signaling cascades [Kasamatsu *et al*. 2008].

The two alternative splice forms were found through BLAST analysis based on the sequence obtained in this study. The longer form encodes 978 amino acids, denoted as GNNK (+), while the shorter form consists of 974 amino acids, denoted as GNNK (-). The two forms of c-Kit mediate distinctively different signals in the hematopoietic cell line Ba/F3. GNNK (-) c-Kit mediates a substantially stronger activation of PI3K/Akt than GNNK (+) c-Kit [Sun *et al*. 2008].

The receptor (c-kit) of SCF is found in two types, i.e. m-kit (membrane-bound kit) and s-kit (soluble form kit). The signaling of SCF and its receptor m-kit plays an important role in melanocyte development, survival, proliferation and melanogenesis. No structure of the fifth Ig-like extracellular domain is found in the s-kit when compared with the m-kit. The SCF/m-Kit signaling is involved in human skin pigmentation and this signaling pathway is regulated by s-Kit [Kasamatsu *et al.* 2008]. There are white or black coat colours in the Taihang goat, an indigenous Chinese breed. In the present study the expression level of the c-*Kit* gene was investigated in two distinct colour variants of the Taihang goat. The mRNA expression of the *c-Kit* gene is greater in skins with the black coat colour than white coat colour, while no significant difference was found in the expression level between them. Further investigation is necessary concerning the genetic mechanism of the *c-Kit* gene in the coat colour of goats.

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