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Full Length Research Paper

Molecular cloning and characterization of two genes - *PHKG2* and *IRAK4* from black-boned sheep (*Ovis aries*)

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The complete coding sequences of two sheep genes-*PHKG2* and *IRAK4* were amplified using the rapid amplification of cDNA ends (RACE) method based on three sheep expressed sequence tags whose translated amino acids contain the mitochondrial carrier domain. The sequence analyses of these two genes revealed that the sheep *PHKG2* gene encodes a protein of 406 amino acids which has high homology with the phosphorylase kinase, gamma 2 protein of five species: Cattle (99%), human (96%), mouse (92%), rat (91%) and chicken (63%). The sheep *IRAK4* gene encodes a protein of 456 amino acids which has high homology with the interleukin-1 receptor-associated kinase 4 protein of six species: Cattle (94%), pig (88%), human (87%), mouse (79%), rat (79%) and chicken (54%). The tissue transcription profile analyses indicated that the Black-boned sheep *PHKG2* and *IRAK4* genes are generally but differentially expressed in the detected tissues including spleen, muscle, skin, kidney, lung, liver, heart, fat and small intestine. These data serve as a foundation for further insight into these two genes.

Key words: Black-boned sheep, *PHKG2* and *IRAK4*, tissue transcription profile.

INTRODUCTION

Phosphorylase kinase gamma 2 (PHKG2) which is phosphorylase kinase catalytic subunit isoform, catalyzes the phosphorylation and activation of glycogen phosphorylase (Winchester et al., 2007). The phosphorylase kinase holoenzyme is made up of four copies of each of four subunits (alpha, beta, gamma and delta). The liver isoforms of the alpha-, beta- and gamma-subunits are encoded by PHKA2, PHKB and PHKG2, respectively. Mutation within these genes has been shown to result in GSD type IX, which is caused by a deficiency of hepatic phosphorylase kinase activity. Seven novel mutations were identified in PHKA2 (p.I337X, p.P498L, p.P869R, p.Y116_T120dup, p.R1070del, p.R916W and p.M113I), two in PHKG2 (p.L144P and p.H48QfsX5) and two in PHKB (p.Y419X and c.2336+965A>C). There was a severe phenotype in patients with PHKG2 mutations, a mild phenotype in patients with PHKB mutations and a broad spectrum associated with PHKA2 mutations (Beauchamp et al., 2007).

Interleukin-1 receptor-associated kinase 4 (IRAK4), is a member of IL-1 receptor (IL-1R)-associated kinase (IRAK) family and has been shown to play an essential role in Toll-like receptor (TLR)-mediated signaling, and IRAK4 kinase activity plays a critical role in TLR-dependent immune responses. The IRAK4 kinase-inactive knock-in mice were completely resistant to lipopolysaccharide (LPS) and CpG-induced shock, due to impairred TLR-mediated induction of proinflammatory cytokines and chemokines. In addition, influenza virus-induced production of interferons in plasmacytoid DCs was also dependent on IRAK4 kinase activity (Kim et al., 2007; De Nardo et al., 2009; Koziczak-Holbro et al., 2007). The crystal structure reveals a six-helical bundle with a prominent loop, which among IRAKs and Pelle, a Drosophila

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Abbreviations: PHKG2, Phosphorylase kinase gamma 2; **IRAK4,** interleukin-1 receptor-associated kinase 4; **RACE,** rapid amplification of cDNA ends; **TLR,** toll-like receptor; **LPS,** lipopolysaccharide; **GSPs,** gene-specific primers; **EST,** expressed sequence tag; **STKc,** serine/threonine protein kinase catalytic domain; **RT-PCR,** reverse transcription- polymerase chain reaction.

Table 1. Primers for sheep *PHKG2*, *IRAK4* and *Actin* genes and their annealing temperatures.

Genes	Primer sequences	Length (bp)	Ta (°C)
PHKG2	Forward: 5'- CCCACTCGGGTGAACTAA -3' Reverse: 5'- AGCTTCATGTTCCTGGTGTT -3'	472	53
IRAK4	Forward: *5'*- CCATCCAAGCAAGCCAGT -*3'* Reverse: *5'*- GTTCAGCAGAAACCGAAGC -*3'*	479	52
Actin	Forward: 5'- AGACCTCTACGCCAACACG -3' Reverse: 5'- ATCCCAGCCTCATAACCCT -3'	419	54

homologue, is unique to IRAK-4. This highly structured loop, contained between helices two and three, comprises an 11-amino acids (aa) stretch. Although innate immune domain recognition is thought to be very similar between Drosophila and mammals, this structural component points to a drastic difference. This structure can be used as a framework for future mutation and deletion studies and potential drug design (Lasker et al., 2005).

The black-boned chicken or silky fowl (*Gallus gallus domesticus brisson*) is the first animal to be shown to possess black muscles and organs (Muroya et al., 2000; Chen et al., 2008; Dorshorst et al., 2010). For thousands of years in oriental societies, the consumption of tissues from black-boned chickens has been believed to confer nutritional and medicinal benefits, improving the immune status and reducing emaciation and feebleness (Nozaki and Makita, 1998; Tian et al., 2007). The discovery of a second animal with black traits, Black-boned sheep *(Ovis aries*) present in a mixed population of sheep found in Nanping County of Yunnan Province, China was reported recently (Deng et al., 2006, 2008a, b). These sheep, like silky fowl, have dark coloured (blackish) tissues, compared to the reddish colour found in normal sheep. The trait for the dark colour in sheep has been shown to be inherited in cross-breeding studies (Deng et al., 2009a, b). Moreover, Black-boned sheep have been certificated as a novel genetic resource by the Ministry of Agriculture, China.

The preceding description of the functions of *PHKG2* and *IRAK4* genes and the association of the genes with growth, health, cell morphology and other important functions that are highly related or potentially related to black traits in the Black-boned sheep, justifies their cloning in the Black-boned sheep.

The objective of this study was to clone and analyze the coding sequences of the Black-boned sheep *PHKG2* and *IRAK4* genes, and determine their tissue transcription profiles. The data obtained will serve as a basis for understanding these sheep genes.

MATERIALS AND METHODS

Samples collection

Six matured Black-boned sheep were slaughtered. Spleen, muscle, skin, kidney, lung, liver, heart, fat and small intestine samples were collected and snap frozen in liquid nitrogen and then stored at -80°C. The total RNA was extracted using the Total RNA Extraction Kit (Gibco, USA). Before the first-strand cDNA synthesis, DNase I treatment of the total RNA was done.

5'- and 3'-RACE

5'- and 3'-RACE were performed to isolate the full-length cDNAs for sheep *PHKG2* and *IRAK4* genes as the instructions of BD SMART™ RACE cDNA Amplification Kit (BD Science, USA). For the sheep *PHKG2* gene, the Gene-Specific Primers (GSPs) were designed based on sheep EST sequence whose translated amino acids contain the partial STKc (Serine/Threonine protein kinases, catalytic) domain: EE799182. 5'-RACE GSP: 5'- CTGGGTGAG TTTCATCCATGGAGCA-3', 3'-RACE GSP: 5'- TGCTCCATGGATG AAACTCACCCAG -3'. For the sheep *IRAK4* gene, the Gene-Specific Primers (GSPs) were designed based on sheep EST sequence whose translated amino acids contain the partial STKc domain: EE805750, 5'-RACE GSP: 5'- GTTCACATAGCCTTTAT ACACAACT-3', 3'-RACE GSP: 5'- CTTTGATGAACGGCCCATTTC TGTC-3'.

These RACE products for sheep *PHKG2* and *IRAK4* genes were then cloned into PMD18-T vector and sequenced bidirectional with the commercial fluorometric method. At least five independent clones were sequenced for every gene.

RT-PCR for tissue expression profile analysis

RT-PCR for tissue expression profile analysis was performed as previously described elsewhere (Liu and Xiong 2007, Liu et al., 2008). We selec-ted the housekeeping gene β-actin (Accession no. NM_001009784) as a positive control. The gene specific primers were used to perform the RT-PCR for tissue expression profile analysis of sheep *PHKG2* and *IRAK4* genes and were listed in Table 1. The 25 µl reaction system was: 2 µl cDNA (100 ng), 5 pmole each oligo-nucleotide primer (forward primer 1 and reverse primer 1 or forward primer and reverse primer 2), 2.5µl 2 mmol/l mixed dNTPs, 2.5µl 10 × Taq DNA polymerase buffer, 2.5µl 25 mmol/l $MgCl_2$, 1.0 units of Taq DNA polymerase, and finally add sterile water to volume 25 µl. The PCR program initially started with a 94°C denaturation for 4 min, followed by 25 cycles of 94°C/ 50s, Ta °C /50s, 72°C /50s, then 72°C extension for 10 min, finally 4°C to terminate the reaction.

Sequence analysis

The gene analysis for cDNA sequence was conducted using Gen-Scan software (http://genes.mit.edu/GENSCAN.html). The protein prediction and analysis were performed using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.

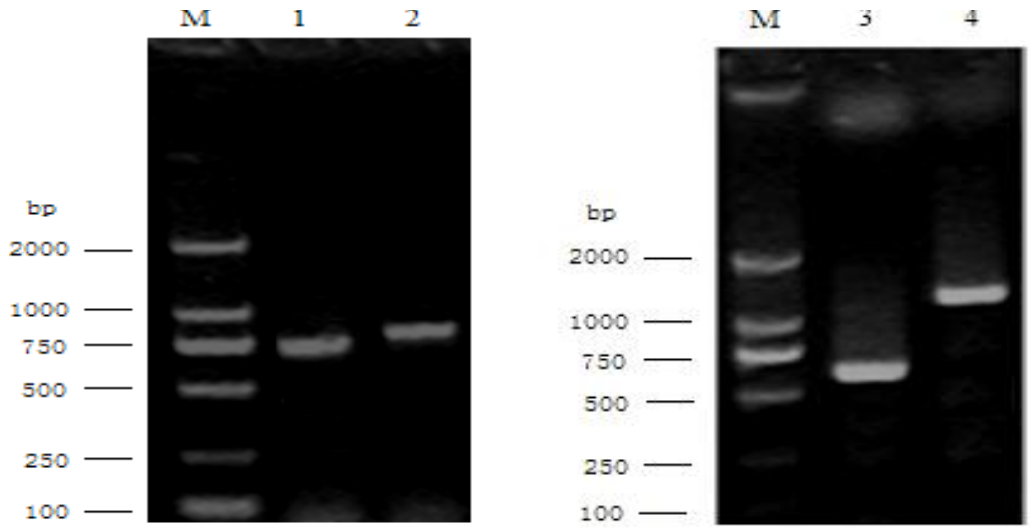

Figure 1. RACE results for sheep *PHKG2* and *IRAK4* genes. M: DL2000 DNA markers; 1: 5'-RACE product for sheep *PHKG2* gene; 2: 3'-RACE product for sheep *PHKG2* gene; 3: 5'-RACE product for sheep *IRAK4* gene; 4: 3'-RACE product for sheep *IRAK4* gene.

nih.gov/BLAST) and the ClustalW software (http://align.genome.jp/). The theoretical isoelectric point (pI) and molecular weight (Mw) of proteins was computed using the Compute pI/Mw Tool (http://www.expasy.org/tools/pi_tool.html). Secondary structures of deduced amino acids sequences were predicted by SOPMA (http://npsa-pbil.ibcp.fr/).

RESULTS

RACE results for sheep *PHKG2* and *IRAK4* genes

For sheep *PHKG2* gene, through 5'-RACE, one PCR product of 749 bp was obtained. The 3'-RACE product was 889 bp. These products were then cloned to T-vector and sequenced. Taken together, a 1613 bp cDNA complete sequence was finally obtained (Figure 1). For sheep *IRAK4* gene, through 5'-RACE, one PCR product of 665 bp was obtained. The 3'-RACE product was 1399 bp. These products were then cloned to T-vector and sequenced. Taken together, a 1982 bp cDNA complete sequence was finally obtained (Figure 1).

Sequence analysis

These cDNA nucleotide sequence analysis using the BLAST software revealed that these genes were not homologous to any of the known sheep genes and they were then deposited into the GenBank database (Accession numbers: FJ422551 and FJ422555). The sequence prediction was carried out using the GenScan software and results showed that the 1613 bp and 1982 bp cDNA sequences represented two single genes which encoded 406 and 456 amino acids, respectively. For sheep *PHKG2* gene, there was a 5' untranslated region of 124 bp and a 3' untranslated region of 278 bp. For sheep *IRAK4* gene, there was a 5' untranslated region of 47 bp and a 3' untranslated region of 564 bp.

The theoretical isoelectric point (pI) and molecular weight (Mw) of these deduced proteins of the two sheep genes were computed using the Compute pI/Mw Tool. The pI of sheep *PHKG2* and *IRAK4* are 5.69 and 5.25, respectively. The molecular weights of these two putative proteins are 46470.32 and 51369.48 dalton, respectively.

These putative proteins were also blasted using the Conserved Domain Architecture Retrieval Tool of Blast at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and their conserved domains were identified as Serine/Threonine protein kinases' catalytic domain (Figure 2).

Further BLAST analysis of these proteins revealed that the sheep *PHKG2* has high homology with the phosphorylase kinase gamma 2 protein of four species: Cattle (NP_001039593, 99%), human (NP_000285, 96%), mouse (NP_081164, 92%), rat (NP_542151, 91%) and chicken (NP_001006217, 63%) (Figure 3). The sheep *IRAK4* has high homology with the interleukin-1 receptor-associated kinase 4 protein of six species: Cattle (NP_001069466, 94%), pig (NP_001106163, 88%), human (NP_057207, 87%), rat (NP_001100261, 79%), mouse (NP_084202, 79%) and chicken (NP_001025909, 54%) (Figure 4).

The prediction of secondary structure by SOPMA (Combet et al., 2000) indicated that the deduced *PHKG2* contained 171 α-helices, 66 extended strands, 37 β-turns and 132 random coils. Furthermore, the deduced *IRAK4* consisted of 187 α-helices, 58 extended strands, 35 β-turns and 176 random coils, respectively (Figure 5).

Based on the results of the alignment of *PHKG2* and *IRAK4*, phylogenetic trees were constructed using the

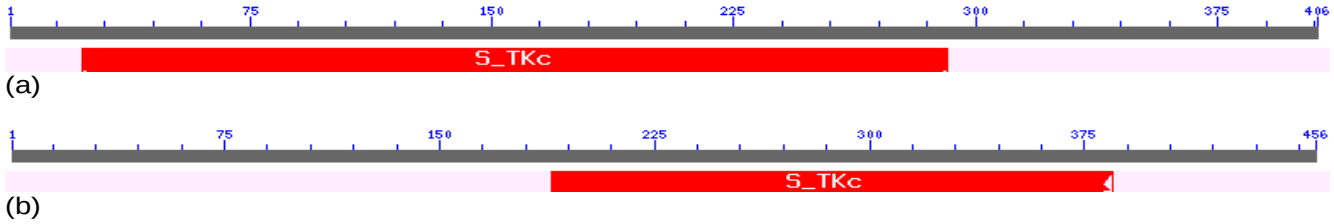

Figure 2. The putative domains of the proteins encoded by sheep *PHKG2* and *IRAK4* gene. (a) Serine/Threonine protein kinases, catalytic domain of sheep PHKG2; (b) Serine/Threonine protein kinases, catalytic domain of sheep IRAK4.

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Sheep           MTLDVGPEDELPDWAAAKEFYQKYDPKDVIGRGVSSVVRRCVHRATGQEFAVKIMEVTAE
Cattle          MTLDVGPEDELPDWAAAKEFYQKYDPKDVIGRGVSSVVRRCVHRATGQEFAVKIMEVTAE
Human           MTLDVGPEDELPDWAAAKEFYQKYDPKDVIGRGVSSVVRRCVHRATGHEFAVKIMEVTAE
Mouse           MTLDVGPEDELPDWAAAKEFYQKYDPKDIIGRGVSSVVRRCVHRATGDEFAVKIMEVSAE
Rat             MTLDVGPEDELPDWAAAKEFYQKYDPKDIIGRGVSSVVRRCVHRATGDEFAVKIMEVSAE
Chicken         ----MTKEEDLPDWNTSKEFYEKYEPKEVLGRGVSSVVRRCIHKATRQEYAVKIIDITAG
                    :  *::***** ::****:**:**:::************:*:** .*:*****::::*

Sheep           RLSPEQLEEVREATRRETHILRQVAGHPHIITLIDSYESSSFMFLVFDLMRKGELFDYLT
Cattle          RLSPEQLEEVREATRRETHILRQVAGHPHIITLIDSYESSSFMFLVFDLMRKGELFDYLT
Human           RLSPEQLEEVREATRRETHILRQVAGHPHIITLIDSYESSSFMFLVFDLMRKGELFDYLT
Mouse           RLSLEQLEEVRDATRREMHILRQVAGHPHIITLIDSYESSSFMFLVFDLMRKGELFDYLT
Rat             RLSLEQLEEVRDATRREMHILRQVAGHPHIITLIDSYESSSFMFLVFDLMRKGELFDYLT
Chicken         NISPQEVQELREATAKEIDILRKVSGHPNVIQLKDSYESSTFFFLVFDLMKRGELFDYLT
                .:* :::::*:*:** :* .***:*:***::* * ******:*:********::********

Sheep           EKVALSEKETRSIMRSLLEAVSFLHNNNIVHRDLKPENILLDDDMQIRLSDFGFSCHLEP
Cattle          EKVALSEKETRSIMRSLLEAVSFLHNNNIVHRDLKPENILLDDNMQIRLSDFGFSCHLEP
Human           EKVALSEKETRSIMRSLLEAVSFLHANNIVHRDLKPENILLDDNMQIRLSDFGFSCHLEP
Mouse           EKVALSEKETRSIMRSLLEAVSFLHANNIVHRDLKPENILLDDNMQIRLSDFGFSCHLEA
Rat             EKVALSEKETRSIMRSLLEAVNFLHVNNIVHRDLKPENILLDDNMQIRLSDFGFSCHLEP
Chicken         EKVTLSEKETRKIMHALLEVIEYLHSIDIVHRDLKPENILLDDDMNIKLTDFGFSCQLHE
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Sheep           GEKLRELCGTPGYLAPEILKCSMDETHPGYGKEVDLWACGVILFTLLAGSPPFWHRRQIL
Cattle          GEKLRELCGTPGYLAPEILKCSMDETHPGYGKEVDLWACGVILFTLLAGSPPFWHRRQIL
Human           GEKLRELCGTPGYLAPEILKCSMDETHPGYGKEVDLWACGVILFTLLAGSPPFWHRRQIL
Mouse           GEKLRELCGTPGYLAPEILKCSMDETHPGYGKEVDLWACGVILFTLLAGSPPFWHRRQIL
Rat             GEKLRELCGTPGYLAPEILKCSMDETHPGYGKEVDLWACGVILFTLLAGSPPFWHRRQIL
Chicken         NEKLKEICGTPGYLAPEILQCSMDDEHEGYGKEVDMWSTGVIMYTLLAGSPPFWHRKQML
                .***:*:************:****: * *******:*: ***::************:*:*

Sheep           MLRMIMEGQYQFSSPEWDDRSDTVKDLISRLLQVDPVERLTAEQALQHPFFERCEGSQAW
Cattle          MLRMIMEGQYQFSSPEWDDRSDTVKDLISRLLQVDPVERLTAEQALQHPFFERCEGSQAW
Human           MLRMIMEGQYQFSSPEWDDRSSTVKDLISRLLQVDPEARLTAEQALQHPFFERCEGSQPW
Mouse           MLRMIMEGQYQFTSPEWDDRSNTVKDLISKLLQVDPEARLTAEQALQHPFFERCEGSQPW
Rat             MLRMIMEGQYQFSSPEWDDRSNTVKDLIAKLLQVDPNARLTAEQALQHPFFERCKGSQPW
Chicken         MLRMIMNGDYQFGSPEWDDRSDTVKDLISQFLVVDPQRRYTAREALAHPFFQQYDVEEVR
                ******:*:*** ********.*******:::* ***  * **.:** ****:: . .:

Sheep           NLTPRQRFRVAVWTVLAAGRVALSAHRIRPLTKSALLRDPYALRPVRRLIDNCAFRLYGH
Cattle          NLTPRQRFRVAVWTVLAAGRVALSAHRIRPLTKSALLRDPYALRPVRRLIDNCAFRLYGH
Human           NLTPRQRFRVAVWTVLAAGRVALSTHRVRPLTKNALLRDPYALRSVRHLIDNCAFRLYGH
Mouse           NLTPRQRFRVAVWTILAAGRVALSSHRLRPLTKNALLRDPYALRPVRRLIDNCAFRLYGH
Rat             NLTPRQRFRVAVWTILAAGRVALSSHRLRPLTKNALLRDPYALRPVRRLIDNCAFRLYGH
Chicken         HFSPFRKFKVICLTVLASVRIYYQYRMVKAVTRELVVRDPYALKPIRKLIDACAFRTYRH
                :::* ::*:*   *:**: *:  . : ::.:*:. ::******:.:*:*** **** * *

Sheep           WVKKGEQQNPAALFQHRLPGPFPMMGPEEEGDSAAIAEDEAMLVLG
Cattle          WVKKGEQQNRAALFQHRPPGPFPMMGPEEEGDSATIAEDEAMLVLG
Human           WVKKGEQQNRAALFQHRPPGPFPIMGPEEEGDSAAITEDEAVLVLG
Mouse           WVKKGEQQNRAALFQHQPPRLFPIAATELEGDSGAITEDEATLVRS
Rat             WVKKGEQQNRAALFQHQPPRPFPIIATDLEGDSSAITEDEVTLVRS
Chicken         WVKKGEAQNRAALFENTCKAVLLTLAAEEELF--------------
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Figure 3. The alignment of the protein encoded by sheep *PHKG2* gene and other five kinds of *PHKG2* from mouse, cattle, rat, human and chicken.

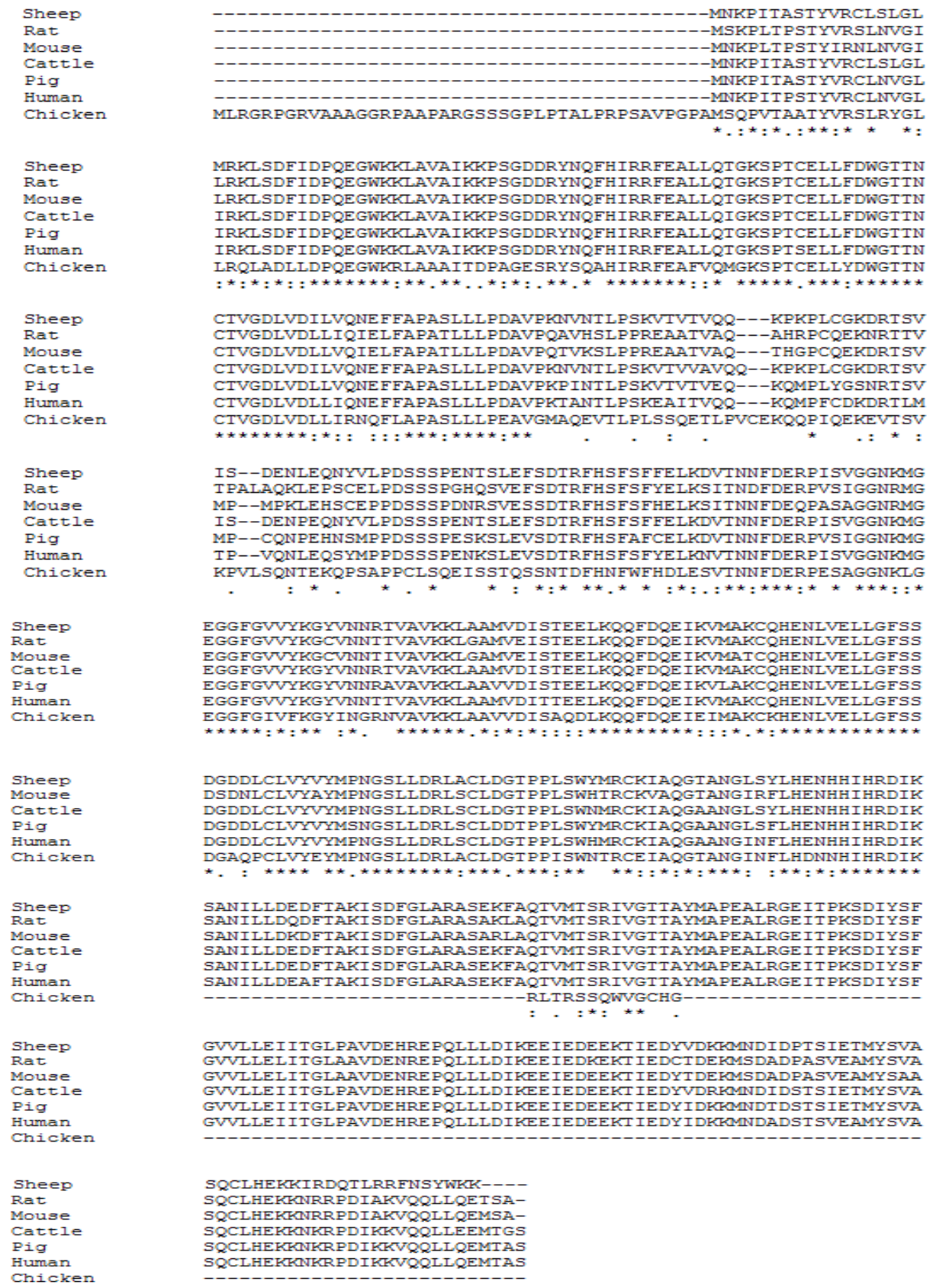

Figure 4. The alignment of the protein encoded by sheep *IRAK4* gene and other six kinds of *IRAK4* from cattle, rat, mouse, pig, human and chicken.

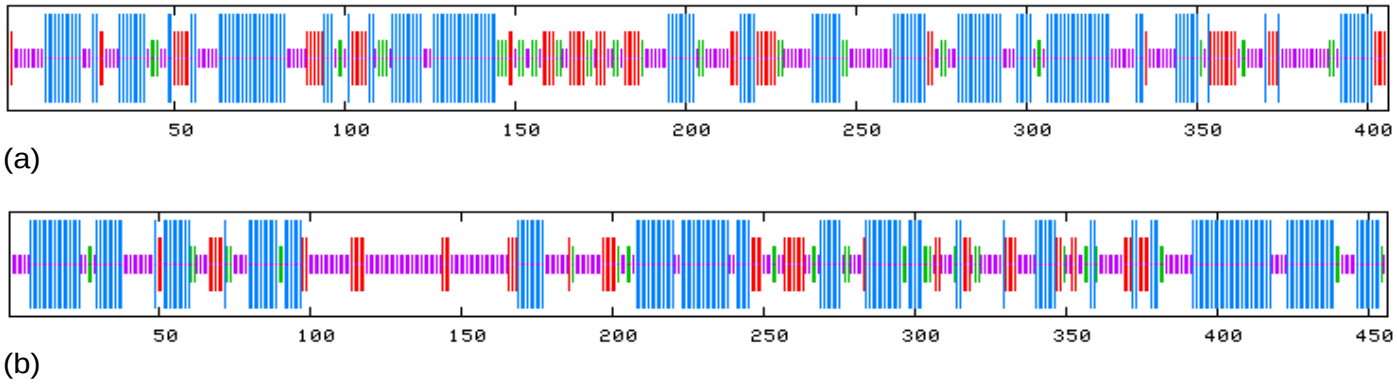

Figure 5. Predicted secondary structure of the Black-boned sheep PHKG2 (a) and IRAK4 (b) proteins by SOPMA. Helices, extended strands, β-turns and random coils are indicated, respectively, with the longest, the second longest, the second shortest and the shortest vertical lines.

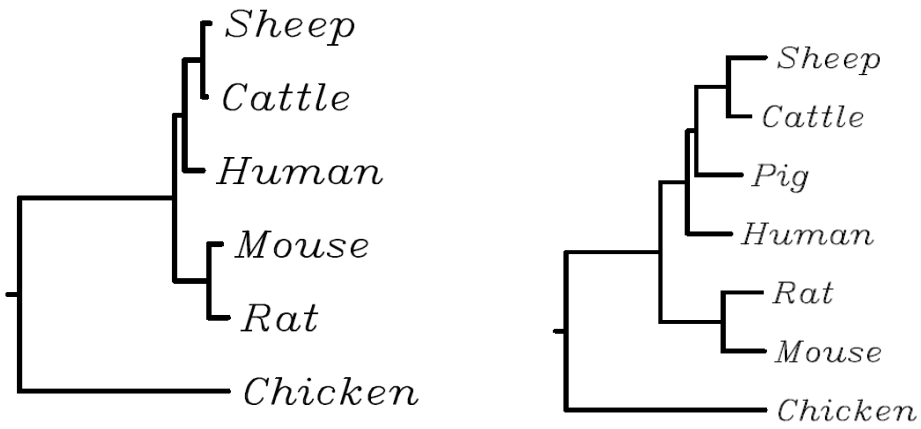

Figure 6. The phylogenetic tree for several kinds of PHKG2 (left) and IRAK4 (right) protein from sheep, cattle, rat, mouse, human and chicken.

Dendrogram procedure of ClustalW software (http://align.genome.jp/), as shown in Figure 6.

The phylogenetic tree analysis revealed that the sheep *PHKG2* has a closer genetic relationship with the *PHKG2* protein of cattle, and sheep *IRAK4* have closer genetic relationships with the *IRAK4* from cattle.

Tissue transcription profile

Tissue transcription profile analysis revealed that, compared to the expression of sheep *actin* gene, the Black-boned sheep *PHKG2* and *IRAK4* genes are generally but differentially expressed in the detected tissues which include; spleen, muscle, skin, kidney, lung, liver and heart, fat and small intestine (Figure 7).

DISCUSSION

In the current study, we firstly get the full length of *PHKG2* and *IRAK4* genes cDNAs and partial 5' and 3'-UTR from the Black-boned sheep (*O. aries*) by using 5'- and 3'- RACE. *PHKG2* and *IRAK4* genes had been reported to play important roles in signal transformation and melanoma, respectively. Thus, this work will provide molecular basis for associate analysis DNA polymorphic of the two genes with black traits of the Black-boned sheep.

The development of modern bioinformatics and specific sheep NCBI EST database was established along with different convenient analysis tools and make researchers much easier to find the useful ESTs which have the important encoding amino acids information (Yang et al., 2009; Yu et al., 2010). Based on their sequences, we can obtain the entire coding sequences through some modern experimental methods such as rapid amplification of cDNA ends (RACE) method. From the isolation and sequence analysis of sheep *PHKG2* and *IRAK4* genes, it could be seen that this is an effective method to isolate the sheep genes.

From the tissue transcription profile analysis in our experiment, it can be seen that these genes were obviously differentially expressed in some tissues. The

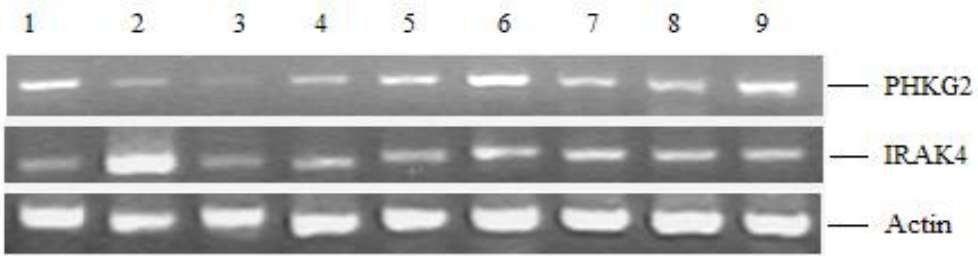

Figure 7. Tissue transcription profile of Black-boned sheep *PHKG2* and *IRAK4* genes. The actin expression is the internal control. 1: spleen; 2: muscle; 3: skin; 4: kidney; 5: lung; 6: liver; 7: heart; 8: fat; and 9: small intestine.

suitable explanation for this is that, at the same time, those biological activities associated with the functions of these genes were presented diversely in different tissues.

Conclusion

We firstly isolated the ovine *PHKG2* and *IRAK4* genes and performed necessary sequence analysis and tissue transcription profile analysis from Black-boned sheep. This established the primary foundation for further insight into the relationship between the polymorphism of these two genes with black traits of Black-boned sheep.

ACKNOWLEDGMENTS

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REFERENCES

Beauchamp NJ, Dalton A, Ramaswami U, Niinikoski H, Mention K, Kenny P, Kolho KL, Raiman J, Walter J, Treacy E, Tanner S, Sharrard M (2007). Glycogen storage disease type IX: High variability in clinical phenotype. Mol. Genet. Metab. 92: 88-99.

Chen SR, Jiang B, Zheng JX, Xu GY, Li JY, Yang N (2008). Isolation and characterization of natural melanin derived from silky fowl (*Gallus gallus domesticus Brisson*). Food Chem. 111: 745-749.

Combet C, Blanchet C, Geourjon C, Deléage G (2000). NPS@: network protein sequence analysis. Trends Biochem. Sci. 25: 147-150.

De Nardo D, Nguyen T, Hamilton JA, Scholz GM (2009). Down-regulation of IRAK-4 is a component of LPS- and CpG DNA-induced tolerance in macrophages. Cell Signal, 21: 246-252.

Deng W, Tan Y, Wang X, Xi D, He Y, Yang S, Mao H, Gao S (2009b). Molecular cloning, sequence characteristics, and polymorphism analyses of the tyrosinase-related protein 2 / DOPAchrome tautomerase gene of black-boned sheep (*Ovis aries*). Genome, 52: 1001-1011.

Deng WD, Xi DM, Gou X, Yang SL, Mao HM (2008a). Physiological, biochemical and genetic profiles of the Black-boned sheep (*Ovis aries*). Pigment Cell Melanoma Res. 21: 266.

Deng WD, Xi DM, Gou X, Yang SL, Shi XW, Mao HM (2008b). Pigmentation in Black-boned sheep (*Ovis aries*): association with polymorphism of the *Tryosinase* gene. Mol. Biol. Rep. 35: 379-385.

Deng WD, Xi DM, Gou X, Yang SL, Shi XW, Mao HM (2009a). Pigmentation in Black-boned sheep (*Ovis aries*): association with polymorphism of the *MC1R* gene. Mol. Biol. Rep. 36: 431-436.

Deng WD, Yang SL, Huo YQ, Gou X, Shi XW, Mao HM (2006). Physiological and genetic characteristics of black-boned sheep (*Ovis aries*). Anim. Genet. 37: 586-588.

Dorshorst B, Okimoto R, Ashwell C (2010). Genomic regions associated with dermal hyperpigmentation, polydactyly and other morphological traits in the Silkie Chicken. J. Hered. doi:10.1093/jhered/esp120.

Kim TW, Staschke K, Bulek K, Yao J, Peters K, Oh KH, Vandenburg Y, Xiao H, Qian W, Hamilton T, Min B, Sen G, Gilmour R, Li X (2007). A critical role for IRAK4 kinase activity in Toll-like receptor-mediated innate immunity. J. Exp. Med. 204: 1025-1036.

Koziczak-Holbro M, Joyce C, Glück A, Kinzel B, Müller M, Gram H (2007). Solving the IRAK-4 enigma: application of kinase-dead knock-in mice. Ernst Schering Found Symp. Proc, pp. 63-82.

Lasker MV, Gajjar MM, Nair SK (2005). Cutting edge: molecular structure of the IL-1R-associated kinase-4 death domain and its implications for TLR signaling. J. Immunol. 175: 4175-4179.

Liu GY, Gao SZ, Ge CR, Zhang X (2008). cDNA cloning and tissue expression analyses of the encoding regions for two novel porcine genes- MJD1, CDC42 and NECD. Anim. Biotechnol. 19: 117-121.

Liu GY, Xiong YZ (2007). Isolation, sequence analysis and expression profile of a novel porcine gene, NIP7, differentially expressed in the Longissimus dorsi muscle tissues from Meishan, Meishan x Large White cross and Large White pigs. Mol. Biol. Rep. 34: 213-219.

Muroya S, Tanabe R, Nakajima I, Chikuni K (2000). Molecular characteristics and site-specific distribution of the pigment of the silky fowl. J. Vet. Med. Sci. 62: 391-395.

Nozaki A, Makita T (1998). Energy dispersive X-ray fluorescence spectrometry of major tissues of silky fowls. J. Vet. Med. Sci. 60: 485-488.

Tian YG, Xie MG, Wang WY, Wu HJ, Fu ZH, Lin L (2007). Determination of carnosine in Black-Bone Silky Fowl (*Gallus gallus domesticus* Brisson) and common chicken by HPLC. Eur. Food Res. Technol. 226: 311-314.

Winchester JS, Rouchka EC, Rowland NS, Rice NA (2007). *In silico* characterization of phosphorylase kinase: evidence for an alternate intronic polyadenylation site in PHKG1. Mol. Genet. Metab. 92: 234-242.

Yang LY, He YD, Kong QB, Zhang WC, Xi DM, Mao HM, Deng WD (2009). Isolation, nucleotide identification and tissue expression of three novel ovine genes-*SLC25A4, SLC25A5* and *SLC25A6*. Mol. Biol. Rep. DOI 10.1007/s11033-009-9812-z.

Yu HM, Chen SN, Xi DM, He YD, Liu Q, Mao HM, Deng WD (2010). Molecular cloning, sequence characterization and tissue transcription profile analyses of two novel genes - *LCK* and *CDK2* from the Black-boned Sheep (*Ovis aries*). Mol. Biol. Rep. 37: 39-45.