aJava

Asian Journal of Animal and Veterinary Advances

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Animal and Veterinary Advances 6 (4): 379-384, 2011
ISSN 1683-9919 / DOI: 10.3923/ajava.2011.379.384

Differential Gene Expression in Different Tissues of Black-Bone Sheep and Normal Sheep

Y. Chen, S.L. Yang, W.D. Deng and H.M. Mao
College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China

Corresponding Author: H.M. Mao, College of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China

ABSTRACT

In order to investigate the molecular mechanism of black-traits in the black-bone sheep, differential mRNA fingerprinting by prerential coding sequences amplification method was used to analyze the differential gene expression in live, heart, pancrease and lumbar muscle between the black-bone sheep and the Lanping native sheep, respectively. Seventy-one expressive sequence tags were obtained between the black-bone sheep and the Lanping native sheep. The results showed that the black-trait of black-bone sheep was controlled by major genes, as well as minor genes based on many differential expressive fragments. and also showed among positive expressive sequence tags in the black-bone sheep, one EST had high similarity (99%) to gluttathion-s-transferase of human (GSTs),

Key words: Sheep, black-bone, eumelanin, DD-PCR, GSTs, EST

INTRODUCTION

The discovery and distribution of Black-bone Sheep were reported firstly and described their living environment, color and anatomy characteristics and the amount of eumelanin deposited in various organisms (Mao *et al*., 2005) and mature black-bone sheep represent the characteristic, the muscle, bone surface (periosteum), kidney, inner skin, heart, lung and trachea of black-bone sheep showed apparently dark color compared with mature normal sheep that grazed in the same pasture (Deng *et al*., 2006). Both black sheep and normal sheep are the same single breeding population (the phylogeny of Lanping indigenous species, 1996). The larger amount of eumelanin was deposited in black-bone sheep, frequently the sheep clearly represented the black traits (Mao *et al*., 2005; Yang *et al*., 2006).

Melanin was distributed widely in various organisms including bacteria, fungi, plants and animals (Wang and Hebert, 2006; Deng *et al*., 2006). It was generally accepted that melanin pigment in human plays an important photoprotective role (Kollias *et al*., 1991; Sarna, 1992; Gilchrest *et al*., 1998) and acted as a cellular antioxidant (Ostrovsky *et al*., 1987) and quench electronically excited states (Sarna *et al*., 1985), as a result melanin protect cells against oxidative stress (Maresca *et al*., 2008). Oxidation of melanin and its irreversible bleaching also followed experimental aerobic irradiation of melanin with ultraviolet or visible light, which leads to the formation of superoxide animo and hydrogen peroxide (Chedekel *et al*., 1978; Korytowski and Sarna, 1990). Melanin pigment is considered as an effective cleaner in that it can change O^- into H_2O_2 (Baldea *et al*., 2009) and reduce damage on biological body and can be a novel medical vector to cure the disease relevant with melanin, such as Parkinsonism (Doublea *et al*., 2009) and

Alzheimer's disease (Wang *et al*., 2006). Melanin play a key role in protecting our skin from damaging effects of UV radiation and in preventing skin cancer (Gray-Schopfe *et al*., 2007). Meanwhile melanin pigment can suppress apptosis induced by flu virus and resist HIV virus (Mandal *et al*., 2005).

MATERIALS AND METHODS

The study began at June 2004, ending at May, 2009.

Animals: A group of healthy sheep including 7 black-boned sheep and 4 Lanpin native sheep were grazed in the same pasture and slaughtered in the same time. All samples were kept fresh and stored in liquid nitrogen.

RNA extraction: Total RNA was isolated from each sample using the Trizol reagent methods (Gibco-BRL, Gaithersburg, MD, USA). Minor DNA among the total RNA was digested using Dnase (Promega, Madison, WI, USA) (Liang and Pardee, 1992). Total RNA of each species was pooled and adjusted to a final concentration of 0. 24 μg μL^{-1}.

RT-PCR: The reaction (20 μL total volume) synthesizing the first cDNA strand contained 0.48 μg total RNA, 50 mm Tris-HCl (pH 8.3), 40 mm KCl, 7 mm $MgCl_2$, 10 mm DTT, 20 mm dNTP and 200 pm of each 3'-end anchored primer (Table 1). The mixture was incubated at 65°C for 5 min, followed by 37°C for 10 min, at which 100 U of M-MLV reverse transcriptase (Promega) was mixed in the reaction was hold at 37°C for 1 h, followed by 75°C for 5 min.

The primers listed in the Table 2 were synthesized and used in the mRNA differential display was described by Fuchs *et al*. (2000). A total of 16 primer combinations (Table 2) were developed, each of which comprised of 4 upper random primers and 4 lower random primers. The temple cDNA was amplified in the total volume of 20 μL by this method. The PCR mixture contained 2 μL of reverse transcription product, 10 mm Tris-HCl (pH 9.0), 50 mM KCL, 1.5 mm $MgCl_2$, 20 mm dNTPs,

Table 1: Primer sequences using for M-MLV reverse transcriptase

Primer	Sequence
H-T_{11}G (2 μM):	5'-AAGCTTTTTTTTTTTG-3
H-T_{11}C (2 μM):	5'-AAGCTTTTTTTTTTTC-3'
H-T_{11}A (2 μM):	5'-AAGCTTTTTTTTTTTA-3'

Table 2: Primer sequences using for mRNA differential display

Primer	Sequence
3'upper primers	
P_1	5'-GGATCCNNNATGA-3'
P_2	5'-GGATCCNNNATGT-3'
P_3	5'-GGATCCNNNATGG-3'
P_4	5'-GGATCCNNNATGC-3'
5'lower random primers	
D_1	5'-GAATTCNNNTCGA-3'
D_2	5'-GAATTCNNNGCGC-3'
D_3	5'-GAATTCNNNGGCC-3'
D_4	5'-GAATTCNNNCCGG-3'

200 pM upper random primers and lower random primers and 1 μL Taq DNA polymerase (Promega). The cycling reaction was denatured at 94°C for 5 min, followed by 35 cycles of 30 sec at 94°C, 60 sec at 40°C, 90 sec at 72°C. The final cycle was followed by an extension step at 72°C for 7 min.

Electrophoresis: The PCR products were separated on 4% denaturing polyacrylamide sequencing gels (0.4 mm thick) in a temperature-regulated Bio-Rad sequencing System (Bio-Rad, Fullerton, CA, USA) at 50°C. Gels were stained with silver and photographed.

To decrease the number of false-positive cDNA fragments, the PCR amplification was repeated twice in the same electrophoretic condition and only selected the fragment between 200 and 1000 bp which were detected in two reactions.

Statistical analysis: The identification of differential displayed fragments was obtained by homology search from the Blast results using the differential expressed fragements from Genebank database in NCBI (the National Center for Biotechnology Information) on http://www.nibi.nih.nlm.gov. Generally, the criteria for scoring a sequence as having a significant match were similarity more than or equal with 70% or E-value less than or equal with $1e^{-10}$ in an overlapping region of at least 70 bp Davoli *et al*. (1999).

Northern blot analysis: Poly A^+RNA was extracted using a Poly (AT) mRNA isolation kit (Progema). Two microliter of each polyA^+ RNA sample was analyzed by electrophoresis through denaturing gels (0.5% agarose/0. 66 M formaldehyde). The gels were blotted using the standard Northern blotting procedures, then hybridized using differential expressed plasmid DNA fragments described above as the probe.

RESULTS

Patterns of differential expressed fragments: A total of 71 cDNA fragments were amplified from four different tissues of black-bone sheep and Lanping native sheep using 16 primer combination comprised of four different 3'-end and four different 5'-end random primers under the same electrophoretic condition. The differential display cDNA fragments showed both quantitative and qualitative differences. For accurately comparing differential display between black-boned sheep and normal sheep, differential fragments expressed cDNA bands fell into two categeories (Fig. 1a, b): 1: bands only detected to appear in black-boned sheep, 2: bans only detected to appear in normal sheep. Part results of alignment can be seen on Table 3.

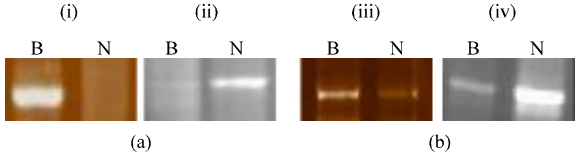

Fig. 1: Patterns of differentially displayed cDNA between black-bone sheep and normal indigious sheep (a) quantitative difference bands and (b) quantitative difference bands. B: Black-bone sheep, N: Lanping native sheep; i: bands detected only in black-bone sheep; ii: bands detected only in Lanping native sheep; iii: bands showing the higher quantitative difference in black-bone sheep than normal indigious sheep; iv: bands showing the higher quantitative difference in Lanping native sheep than black-bone sheep. Blast of sequences was completed on http://www.nibi.nih.nlm.gov/blast

Table 3: The sequencing and alignment part results of differential expressed fragments

DDFN	Source of tissue	Result of hybridization	Homology of No.	Prediction of function
DDF1	Pancrease	B	gi|601450|gb|T19407.1|	Glutathione
DDF2	Liver	B	gi|14414962|gb|BI010891.1|	Tumor inhibitor
DDF3	Heart	B	gi|14414971|gb|BI010900.1|	Tumor inhibitor
DDF4	Liver	B		unknown
DDF5	Liver	B	gi|32044967|emb|BX508100.1|	Relation of cancer
DDF6	Lumber	N		unknown
DDF7	Pancrease	B	gi|14414962|gb|BI010891.1|	Inhibitating the tumor
DDF8	Pancrease	B	gi|14414971|gb|BI010900.1|	Tumor inhibitative

DDFN: Differential displayed fragments of No. B: Bands ouly detected in black-bone sheep bot not detected in normal indigious sheep through the Northern blot hybrization. N: Bands ouly detected in normal indigenious sheep but not detected in black-bone sheep through the Northern blot hybrization. Homology of No.: The greatest similar sequences of No. in the Genbank of NCBI found through using the Blast software

DISCUSSION

Experimental data presented in this study demonstrated that there were differential expression genes between black-bone sheep and Lanping native sheep, as a result the black-trait character of black-bone sheep was controlled by major genes, as well as minor genes, which is probably relevant with the formation of the melanin and affact the deposition of the amount of melanin in the body of black-bone sheep. The same result represented in the study of black spotting in pigs (Kijas *et al*., 2001), the human beings (Reid *et al*., 1996) and mouse pigment (Jackson *et al*., 1994).

Four differential fragments (DDF2, DDF5, DDF7, DDF8) have high homology with genes related with the cancer and tumor. Previous studies shows that melanocytes have the key role in preventing skin cancer (Gray-Schopfer *et al*., 2007) and the presence of melanin pigment is a valuable indicator with which to justify the diagnosis of melanoma, cell melanoma can provide useful clues to tumor origin (Piao *et al*., 2007; Quintana *et al*., 2008).

The differential fragment No. 1 DDF1 have most homology with the Glutathione S transferase (Expect = 2e-49, Identities = 498/504 (99%)), based on the former research, the greater amount of eumelanin were deposited in the black-bone sheep than in Lanping native sheep (Yang *et al*., 2006). The procession of melain synthesis begins with the tyrosinase catalysed conversation of tyrosine to the dopaquinone. the spontaneous conversation of dopaquinone to 3, 4-dihydroxyphenylanineor dopachrome (Aroca *et al*., 1992). As a result, both eumelanin and pheeomelanin are dedrived from the common precursor dopaquinone that is formed by tyrosinase oxidation of tyrosine. The glutathione-S-transferase acting as highly reactive intermediate play the role in the transferation from dopaquinone and in the absent of thiol compounds it undergoes intramolecular cylization, leading eventually to the products of eumelanin. However, intervention of thiols such as cyteine with its process give rise exclusively to thiols adducts of Dopa, termed cycleinydopas, among which 5-s-cysteinyldopa is major isomer (Landand Riley, 2000). The glutathione-S-transferase transfer the-SH to develop and form the dopachrome and the larger amount of eumelanin was deposited in black-bone sheep, as a frequent the sheep clearly represented the black traits.

ACKNOWLEDGMENTS

The author thank the State Key Lab of Agrobiotechnology, China, especially professor Ning Li, Xiaoxiang Hu for expert technical assistance. This work was funded by Key Projects in the National

Science and Technology Pillar Program During the Eleventh Five-year Plan Period (grant No. 2008BADB2B01) and National Hi-New Technology Research and Development Plan of China (grant No. 2008AA101001).

REFERENCES

Aroca, P., F. Solano, C. Salinas, J.C. Garcia-Borron and J.A. Lozano, 1992. Regulation of the final phase of mammalian melanogenesis. The role of dopachrome tautomerase and the ratio between 5,6-dihydroxyindole-2-carboxylic acid and 5,6-dihydroxyindole. Eur. J. Biochem., 208: 155-163.

Baldea, I., T. Mocan and R. Cosgarea, 2009. The role of ultraviolet radiation and tyrosine stimulated melanogensis in the induction of oxidative stress alternations in fair skin melanocytes. Exp. oncol., 31: 200-208.

Chedekel, M.R., S.K. Smith, P.W. Post, A. Pokora and D.L. Vessel, 1978. Photodestruction of pheomelanin: Role of oxygen. Proc. Nat. Acad. Sci. USA., 75: 5395-5399.

Davoh, R., P. Zambonelli, D. Bigi, L. Fontanesi and V. Russo, 1999. Analysis of expressed sequence tags of porcine skeletal muscle. Gene, 233: 181-188.

Deng, W.D., S.L. Yang, Y.Q. Huo, X. Gou, X.W. Shi and H.M. Mao, 2006. Physiological and genetic characteristics of black-boned sheep(*Ovis aries*). Anim. Genet., 37: 586-588.

Doublea, K.L., D.B. Rowe, F.M. Carew-Jonesa, M. Hayesc and D.K.Y. Chan *et al*., 2009. Anti-melanin antibodies are increased in sera in Parkinson's disease. Exp. Neurol., 217: 297-301.

Fuchs, B., K. Zhang, M.E. Bolander and G. Sarkar, 2000. Differential mRNA fingerprinting by preferential amplification of coding sequences. Gene, 258: 155-163.

Gilchrest, B.A., H.Y. Park, M.S. Eller and N. Yaar, 1998. The Photobiology of the Tanning Response. In: The Pigmentary System. Physiology and Pathophysiology, Nordlund, J.J., R.E. Boissy, V.J. Hearing, R.A. King and J.P. Ortonne (Eds.). Oxford University Press, New York, pp: 359-372.

Gray-Schopfe, V., C. Wellbrock and R. Marais, 2007. Melanoma biology and new targeted therapy. Nature, 445: 851-857.

Jackson, I.J., P. Budd, M.H. Jacqueline, R. Johnson, S. Raymond and K. Steel, 1994. Genetics and molecular biology of mouse pigmentation. Pigment Cell Res., 7: 73-80.

Kijas, J.M., M. Moller, G. Plastow and L. Andersson, 2001. A frameshift mutation in MC1R and a high frequency of somatic reversions cause black spotting in pigs. Genetics, 158: 779-785.

Kollias, N., R.M. Sayre, L. Zeise and M.R. Chedekel, 1991. New trends in photobiology: Photoprotection by melanin. Photochem. Photobiol. Biol., 9: 135-160.

Korytowski, W. and T. Sarna, 1990. Bleaching of melanin pigments: Role of copper ions and hydrogen peroxide in autooxidation of synthetic dopa-melanin. J. Biol. Chem., 265: 12410-12416.

Land, E.J. and P.A. Riley, 2000. Spontaneous redox reaction of dopaquinane and balance between the eumelanic and phaemelanic pathic pathways. Pigment Cell Res., 13: 273-277.

Liang, P. and A.B. Pardee, 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science, 257: 967-971.

Mandal, P., U. Baneriee, A. Casadeval and J.D. Nosanchuk, 2005. Dual infections with pigmented and albino strains of crypoccus neoformans in patients with or without human immuno deficiency virus infection in India. J. Clin. Microbiol., 43: 4766-4772.

Mao, H.M., W.D. Deng, S.R. Shun, W. Shu and S.L. Yang, 2005. Studies on the specific characteristics of Yunnan Black-bone sheep. J. Yunnan Agric. Univ., 20: 89-93.

Maresca, V., E. Flori, S. Briganti, A. Mastrofrancesco and C. Fabbri *et al.*, 2008. Correlation between melanogenic and catalase activity in *in vitro* human melanocytes: A synergic strategy against oxidative stress. Pigment cell melanoma Res., 21: 200-205.

Ostrovsky, M.A., N.L. Sakina and A.E. Dontsov, 1987. An antioxidative role of ocular screening pigments. Vision Res., 27: 893-899.

Piao, Y., M. Gao and Y. Gong, 2007. Diagnostic challeges of metastatic spindle cell melanoma on fine-needle Aspiration specimens. Cancer, 114: 94-101.

Quintana, E., M. Shacketon, M.S. Sabel, D.R. Fullen, T.M. Johnson and S.J. Morrison, 2008. Efficient tumour formation by single human melanoma cells. Nature, 456: 593-598.

Reid, K., A.M. Turnley, G.D. Maxwell, Y. Kurihara, H. Kurihara, P.F. Bartlett and M. Murphy, 1996. Multiple roles for endothelin in melanocyte development: Regulation of progenitor number and stimulation of differentiation. Development, 122: 3911-3919.

Sarna, T., I.A. Menon and R.C. Sealy, 1985. Photosensitization of melanins: A comparative study. Photochem. Photobiol., 42: 529-532.

Sarna, T., 1992. Properties and function of the ocular melanin-a photobiophysical view. J. Photochem. Photobiol., 12: 215-258.

Wang, N. and D.N. Hebert, 2006. Tyrosinase maturation through the mammalian secretary pathyway: Bringing color to life. Pigment Cell Res., 19: 3-18.

Wang, R., P. Tang, P. Wang, R.E. Boissy and H. Zheng, 2006. Regulation of tyrosinase trafficking and processing by presenihns:partial loss of function by familial Alzheimer's disease mutation. Proc. Nat. Acad. Sci. USA., 103: 353-358.

Yang, S.L., H.M. Mao, W. Shu and W.D. Deng, 2006. Melanin Traits of yunnan black-bone sheep and TYR gene polymorphism. Yi Chuan, 28: 291-298.