pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2015) Vol. 30, No. 2, pp. 99~107
http://dx.doi.org/10.12750/JET.2015.30.2.99

Expression of Coat Color Associated Genes in Korean Brindle Cattle by Microarray Analysis

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ABSTRACT

The aim of the present study was to identify coat color associated genes that are differentially expressed in mature Korean brindle cattle (KBC) with different coat colors and in Hanwoo cows. KBC calves, before and after coat color appearance, were included. Total cellular RNA was isolated from the tail hair cells and used for microarray. The number of expressed coat color associated genes/probes was 5813 in mature KBC and Hanwoo cows. Among the expressed coat color associated genes/probes, 167 genes were the coat color associated genes listed in the Gene card database and 125 genes were the pigment and melanocyte genes listed in the Gene ontology_bovine database. There were 23 genes/probes commonly listed in both databases and their expressions were further studied. Out of the 23 genes/probes, MLPH, PMEL, TYR and TYRP1 genes were expressed at least two fold higher (p<0.01) levels in KBC with brindle color than either Hanwoo or KBC with brown color. TYRP1 expression was 22.96 or 19.89 fold higher (p<0.01) in KBC with brindle color than either Hanwoo or KBC with brown color, respectively, which was the biggest fold difference. The hierarchical clustering analysis indicated that MLPH, PMEL, TYR and TYRP1 were the highly expressed genes in mature cattle. There were only a few genes differentially expressed after coat color appearance in KBC calves. Studies on the regulation and mechanism of gene expression of highly expressed genes would be next steps to better understand coat color determination and to improve brindle coat color appearance in KBC.

(Key words : Korean brindle cattle (Chikso), coat color, gene expression, microarray)

INTRODUCTION

Genetics of coat color in animals (Barsh, 2001) and in cattle (Olson, 1999) has been reviewed. Synthesis of eumelanin and phaeomelanin are important in coat colors of cattle. *Tyrosinase related protein 1* (*TYRP1*) affects coat colors with eumelanin synthesis (Berryers *et al.*, 2003). *Melanocortin 1 Receptor* (*MC1R*) influences eumelanin and phaeomelanin synthesis, and *MC1R* genotypes are responsible for most variation of cattle coat color in many breeds including Hanwoo and KBC (Klungland *et al.*, 1995; Chung *et al.*, 2000; Berryers *et al.*, 2003; Do *et al.*, 2007; Lee *et al.*, 2008; Park *et al.*, 2012). *Agouti signaling protein* (*ASIP*) is the natural antagonist of *MC1R* (Barsh, 1996). Investigators have identified other genes that affect coat colors of cattle including *tyrosinase* (*TYR*) (Schmutz *et al.*, 2004), *KIT* (Reinsch *et al.*, 1999) and *KIT ligand* (*KITLG*) (Charlier *et al.*, 1996; Seitz *et al.*, 1999). Schmutz *et al.* (2013) reported an interaction between the *MC1R* and *premelanosomal protein* (*PMEL*). Brenig *et al.* (2013) investigated the genetic background of mismarked coat color in White Galloway cattle, where mating of individuals with the preferred well or strongly marked phenotype also resulted in offspring with the undesired mismarked and/or even fully black coat color in White Galloway cattle. The results of this study indicated that the white coat color variation was caused by the ploidy of aberrant insertions and inheritance of the *KIT* gene on chromosome 29 in White Galloway and White Park cattle. Data suggests that numerous genes are involved in the coat color appearance in cattle, and expression of specific coat color associated genes may be important in certain breeds of cattle. The objective of this study was to determine the gene expression patterns by microarray 1) in mature KBC with different coat colors and in Hanwoo cows, and 2) in KBC calves before and after the brindle coat color appearance.

MATERIALS AND METHODS

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1. Experimental Animals and RNA Extraction

Total number of experimental animals used in this study were 20, including 4 Hanwoo cows and 16 Koran brindle cattle (KBC). KBC included 12 mature cattle with 3 different coat colors, and 4 calves between 1 to 2 months of age. Hanwoo had brown coat color, while mature KBC had either whole brindle, brown or black coat color. Whole brindle was defined as having brindle coat more than 50% of body, brown as having similar coat color as Hanwoo, and black as having black coat all of the body (Park *et al.* 2012). Four KBC calves were included to determine the changes of gene expression before and after the brindle coat color appearance.

For microarray analysis, RNA was extracted from tail hair cells of brindle, brown or black coat color of KBC and from tail hair cells of brown coat color of Hanwoo. For KBC with brindle coat color, hair cells from brindle area with mainly dark brown and black hairs were used. Less than 5 mg of material was inserted into the 1.5 ml microcentrifuge tube and total cellular RNA was extracted using RNeasy Micro Kit (Qiagen, Germany). Quality of RNA was determined by measuring absorbance at 260 nm/280 nm and 260 nm/230 nm, and 28s/18s RNA ratio using a 2100 Bioanalyzer (Agilent Technologies, USA). RNA integrity number (RIN) was calculated for each sample using Agilent 2100 Expert software, and RNA was stored frozen at −70℃ until analysis.

2. Microarray Analysis

To compare the gene expression pattern between the experimental groups, the procedure of amplification, labeling and hybridization was conducted using Agilent's Low Input Quick Amp Labeling kits (Agilent Technology, USA) and Agilent bovine 4×44K chip, following the manufacture's protocol. One μg of total cellular RNA was mixed with T7 promoter primer and was incubated at 65℃ for 10 min. Then, cDNA master mix (5X First strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and MMLV-RT) was prepared and added into the microcentrifuge tube. Samples were incubated at 40℃ for 2 hr. Following reverse transcription and dsDNA synthesis, transcription master mix (4X Transcription buffer, 0.1 M DTT, dNTP mix, 50% PEG, RNase-Out, inorganic pyrophosphatase, T7-RNA polymerase, and cyanine 3-CTP) was prepared, added to dsDNA tubes, and incubated at 40℃ for 2 hr. Labeled cRNA was purified and quantified with ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). Labeled cRNA was then fragmented, mixed with hybridization buffer, and placed on the microarray slide. The microarrays were then incubated at 65℃ for 17 hr in a hybridization oven (Agilent Technology, USA) and washed. Dried microarray slides were scanned using Agilent DNA microarray Scanner (Agilent Technology, USA). The images obtained after scanning were analyzed with Agilent Feature Extraction software (Agilent Technology, USA). Fluorescent intensity from each spot was calculated after background subtraction. All data normalization and selection of fold-changed genes were performed using GeneSpringGX 7.3.1 program (Agilent Technology, USA).

RESULTS

1. Gene Expression in Korean Brindle Cattle with Different Coat Colors and in Hanwoo

Microarray analysis was performed with the Agilent bovine 4×44K chip to compare the coat color gene expression in mature Korean brindle cattle (KBC) with different coat colors and in Hanwoo cows. We identified 5813 expressed genes/probes that were related to coat color, pigmentation, melanocyte, and melanogenesis in the hair cells of mature cattle. One hundred sixty seven genes expressed in the microarray were coat color associated genes listed in the Gene card database and 125 genes expressed in the microarray were bovine pigment and melanocyte genes listed in the Gene ontology_bovine database. There were 23 genes/probes expressed in the microarray, which were listed both in Gene card database and Gene ontology_bovine database (Table 1).

Expressions of 167 coat color associated genes from different experimental groups were compared by scatter plots (Fig. 1) and the expression levels of the 23 selected genes/probes in Table 1 were further investigated. When Hanwoo and KBC with brown coat color was compared, the expressions of ASIP, DCT, LEF1 (LYMPHOID ENHANCER-BINDING FACTOR 1), and SLC45A2 genes were at least two fold higher ($p<0.01$) in KBC with brown coat color than in Hanwoo (Fig. 1A). LEF1 was not listed in Table 1. The difference was the most in DCT expression with 6.28 fold higher ($p<0.01$) in KBC with brown coat color than in Hanwoo. The expressions of ASIP, EDA, MLPH, PMEL, SLC45A2, TYR, and TYRP1 genes were at least two fold higher ($p<0.01$) in KBC with brindle coat color than in Hanwoo (Fig. 1B). EDA (ECTODYSPLASIN A) and SLC45A2 (SOLUTE CARRIER FAMILY

Table 1. List of 23 bovine coat color associated genes and pigment/melanocyte genes or probes

Gene symbol	Gene name	GenBank accession	Probe ID
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	NM_001166484	A_73_P087626
MLPH	Melanophilin	NM_001081597	A_73_P317496
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	NM_001166484	A_73_113522
PMEL	Premelanosome protein	NM_001080215	A_73_P036316
TYR	Tyrosinase (oculocutaneous albinism IA)	NM_181001	A_73_P251106
MC1R	Melanocortin 1 receptor (alpha melanocyte stimulating hormone receptor)	NM_174108	A_73_P036956
TYR	Tyrosinase (oculocutaneous albinism IA)	NM_181001	A_73_P037571
ASIP	Agouti signaling protein	NM_206843	A_73_P437651
ASIP	Agouti signaling protein	NM_206843	A_73_107408
DCT	Dopachrome tautomerase	NM_001012666	A_73_P040651
TYRP1	Tyrosinase-related protein 1	NM_174480	A_73_P041341
EDNRB	Endothelin receptor type B	NM_174309	A_73_P377566
EDNRB	Endothelin receptor type B	AB099006	A_73_P099906
EDNRB	Endothelin receptor type B	NM_174309	A_73_P038281
ARCN1	Archain 1	NM_001195013	A_73_P075116
KITLG	KIT ligand	NM_174375	A_73_110108
TYRP1	Tyrosinase-related protein 1	NM_174480	A_73_105798
MLPH	Melanophilin	NM_001081597	A_73_107222
TYRP1	Tyrosinase-related protein 1	NM_174480	A_73_P411006
PMEL	Premelanosome protein	M81193	A_73_107037
KITLG	KIT ligand	NM_174375	A_73_P051146
MREG	Melanoregulin	NM_001192953	A_73_P057721
DCT	Dopachrome tautomerase	NM_001012666	A_73_102308

45, MEMBER 2) were not listed in Table 1. The difference was the most in TYRP1 expression with 22.96 fold higher, followed by PMEL expression with 5.80 fold higher, in KBC with brindle coat color ($p<0.01$) than in Hanwoo. The expressions of DCT, EDA, MLPH, PMEL, SLC45A2, SOX10, and TYR genes were at least two fold higher ($p<0.01$) in KBC with black coat color than in Hanwoo (Fig. 1C). EDA, SLC45A2, and SOX10 (SRY-BOX 10) were not listed in Table 1. The difference was the most in TYRP1 expression with 28.97 fold higher, followed by PMEL and TYR expressions with 7.29 and 7.42 fold higher, respectively, in KBC with black coat color ($p<0.01$) than in Hanwoo.

When KBC with brindle coat color and KBC with brown coat color was compared, the expressions of CITED1, MLPH, NF1, PMEL, TYR, and TYRP1 genes were at least two fold higher ($p<0.01$) in KBC with brindle coat color than in KBC with brown coat color (Fig. 1D). CITED1 (CBP/p300-INTERACTING TRANSACTIVATOR WITH GLU/ASP-RICH C-TERMINAL DOMAIN, 1) and NF1 (NEUROFIBROMATOSIS, TYPE I) genes were not listed in Table 1. The difference was the most in TYRP1 expression with 19.89 fold higher, followed by MLPH expression with 4.13 fold higher, in KBC with brindle coat color ($p<0.01$) than in KBC with brown coat color. The expressions of MLPH, PMEL, SOX10, TYR, and TYRP1

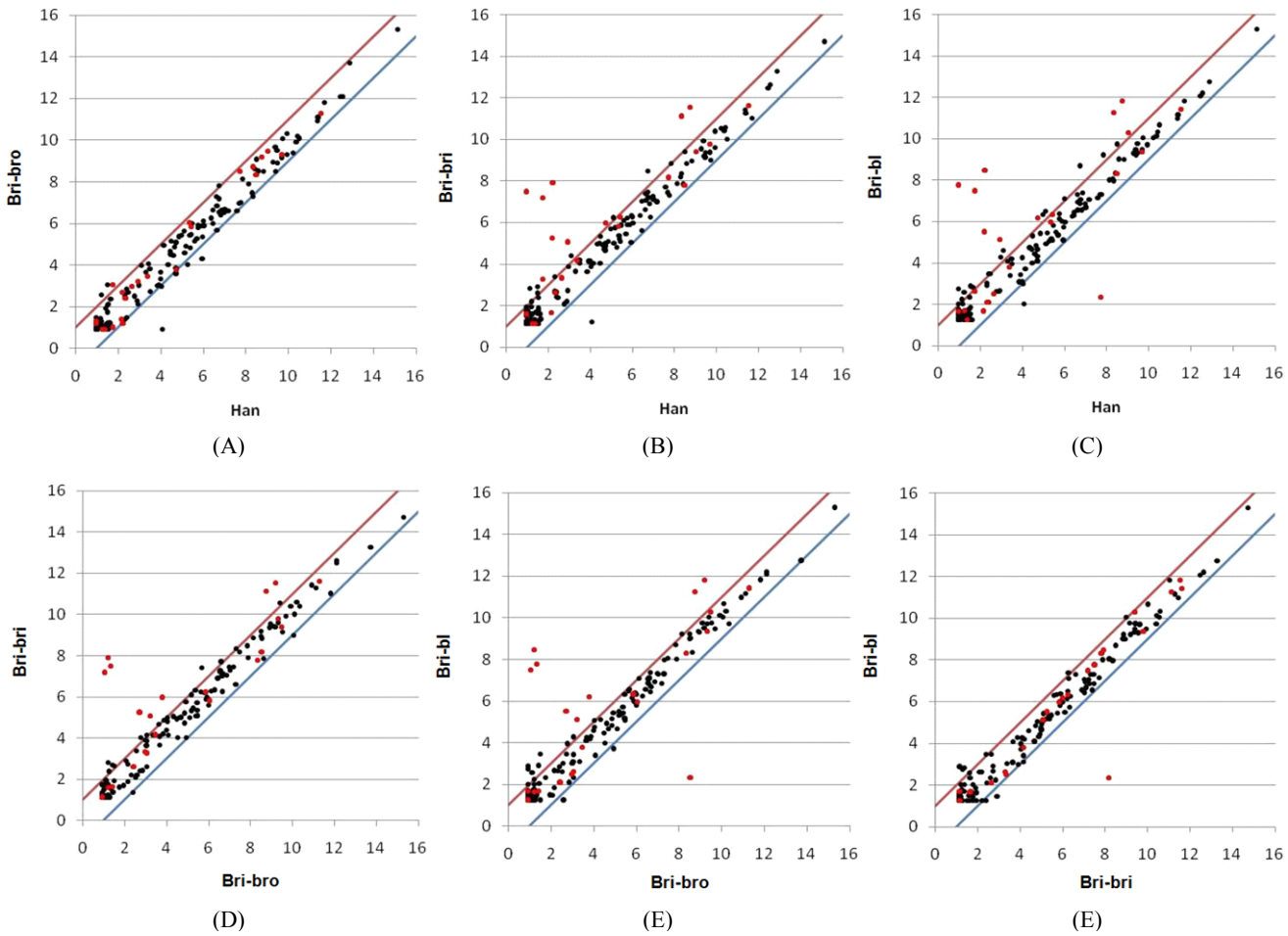


Fig. 1. Scatter plot of selected coat color associated genes in Korean brindle cattle and Hanwoo cows. Scatter plots displaying the expression patterns of selected genes by fold differences between the experimental groups among Korean brindle cattle with 3 different coat colors and Hanwoo are shown. Black spots represents 167 expressed genes that are listed as coat color related genes in Gene card database and red spots represents 23 expressed genes/probes that are listed as pigment and melanocyte related genes in database of Gene ontology_bovine as well as coat color related genes in Gene card database, respectively. Spots located outside the two diagonal red and blue lines indicate more than 2-fold gene expression differences. Some spots were placed on top of each other depicting less than the total number. Han : Hanwoo with brown coat color, Bri-bro : Korean brindle cattle with brown coat color, Bri-bri : Korean brindle cattle with whole brindle (≧50%) coat color, Bri-bl : Korean brindle cattle with black coat color.

genes were at least two fold higher (p<0.01) in KBC with black coat color than in KBC with brown coat color (Fig. 1E). SOX10 was not listed in Table 1. The difference was the most in TYRP1 expression with 25.09 fold higher, followed by TYR and MLPH expressions with 5.20 and 5.03 fold higher, respectively, in KBC with black coat color (p<0.01) than in KBC with brown coat color. The expression of only LOC785371 was at least two fold higher (p<0.01) in KBC with black coat color than in KBC with brindle coat color (Fig. 1F). LOC785371 (Uncharacterized protein, related to GLI-KRUPPEL FAMILY MEMBER 3; GLI3) was not listed in Table 1.

In the hierarchical clustering analysis of the contrasts between two experimental groups, there were three clusters (Fig. 2). The first cluster included Bri-bri/Han and Bri-bri/Br-bro contrasts, the second cluster Bri-bl/Han and Bri-bl/Bri-bro contrasts, and third cluster Bri-bro/Han and Bri-bl/Bri-bri contrasts. The first and the second clusters were linked first, and then with the third cluster. In the hierarchical clustering analysis of coat color genes, there were four clusters. TYRP1 gene was in the first cluster, while MLPH, TYR, and PMEL genes were in the

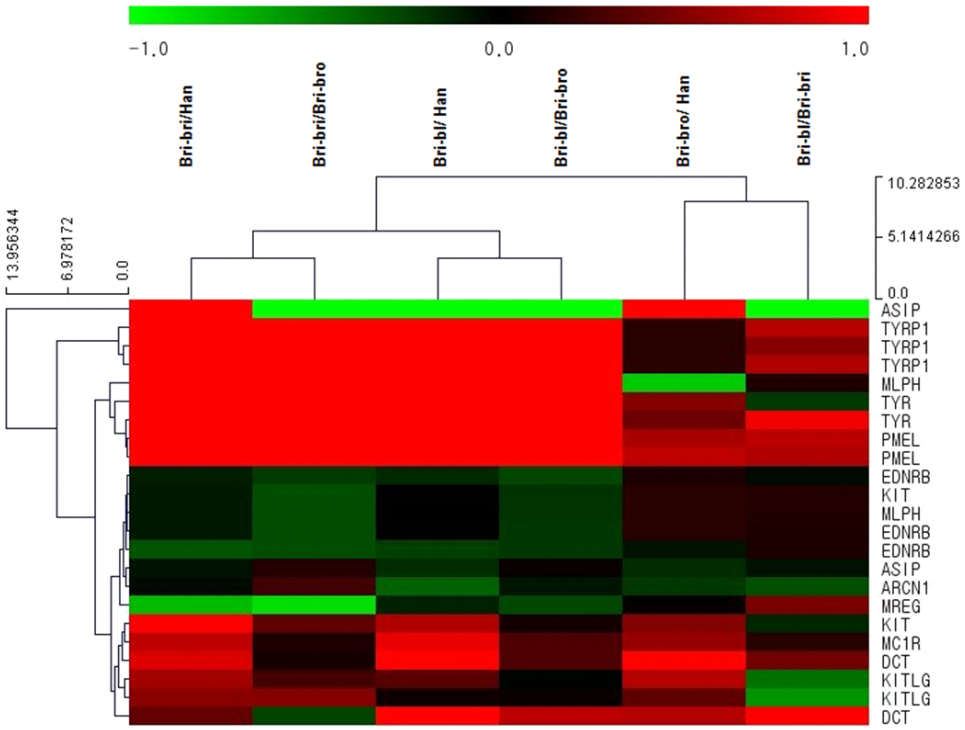


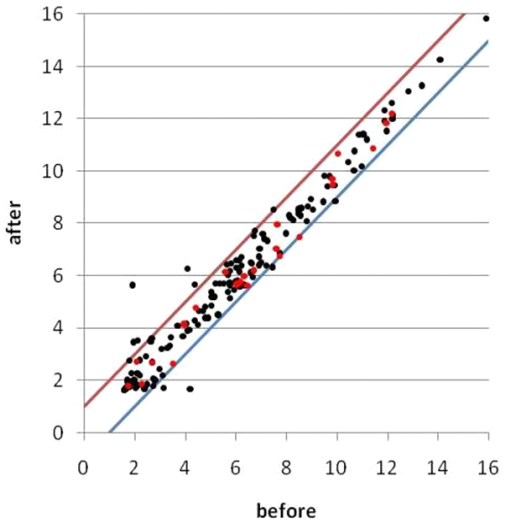
Fig. 2. Hierarchical clustering analysis of experimental group contrasts and 23 selected genes/probes. Hierarchical clustering analysis display clusters among contrasts between two experimental groups and also the expression patterns of the 23 selected genes/probes in Korean brindle cattle with 3 different coat colors and in Hanwoo. The red color indicates the up-regulated genes and green color indicates the down-regulated genes. Han : Hanwoo with brown coat color, Bri-bri : Korean brindle cattle with whole brindle (≧50%) coat color, Bri-bro : Korean brindle cattle with brown coat color, Bri-bl : Korean brindle cattle with black coat color.

second cluster. Gene expression in these two clusters was high. EDNRB, KIT, MLPH, ASIP, ARCN1 and MREG genes were in the third cluster, and their expression was low. KIT, MC1R, DCT and KITLG genes were in the fourth cluster, and their expression was relatively high. The last two clusters were linked first, followed by the second cluster, and the first cluster. ASIP was not in the cluster with other genes and linked last with the rest of the clusters.

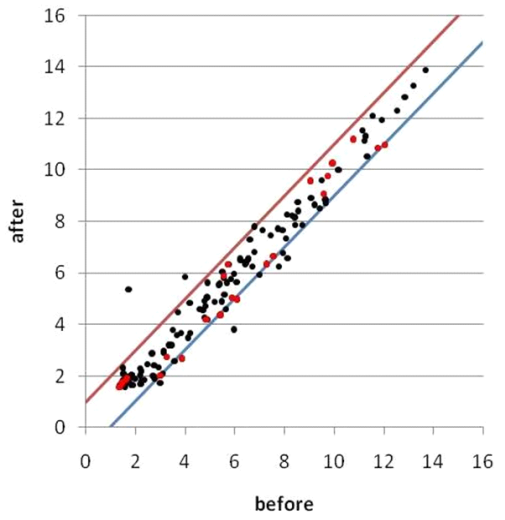
2. Gene Expression in Korean Brindle Cattle Calves before and after Coat Color Expression

Four Korean brindle cattle (KBC) calves were included to determine the gene expression patterns before and after the coat color appearance. The coat colors of two KBC calves started to change from brown to brindle between 27 to 48 days of age, while the other two calves maintained the same brown coat color. Expressions of 167 coat color associated genes were compared before and after the color changes by scatter plots (Fig. 3) and the expression levels of the 23 selected genes/probes in Table 1 were further investigated. No genes from Table 1 were expressed more than 2 fold after coat color determination in KBC calves with brown coat color (Fig. 3A). Though not listed in Table 1, expression of ZIC2 (ZINC FINGER PROTEIN OF CEREBELLUM 2) gene was 17.0 fold higher (p<0.01) after coat color determination than before. In KBC with brindle coat color, the expression of TYRP1 gene was 2.18 fold higher (p=0.02) after brindle coat color appearance than before (Fig. 3B). Expression of ZIC2 gene was 14.9 fold higher (p=0.01) after brindle coat color appearance than before.

In the hierarchical clustering analysis, there were no clusters between the KBC calves with brown or brindle coat color (Fig. 4). In principle, gene expression was higher in KBC calves with brindle coat color than in KBC calves with brown coat color. For coat color gene expressions, clustering was more complicated in the KBC calves than the experimental group contrasts of mature cattle. In the first cluster, there were KIT, KITLG, ASIP, and MREG genes in a sequential manner. Their expression was low in both KBC calves with brown coat color



(A)



(B)

Fig. 3. Scatter plot of selected coat color associated genes/probes in KBC calves before and after the coat color change. Scatter plots displaying the expression patterns of selected genes by fold differences before and after the coat color change in Korean brindle cattle (KBC) with brown coat color (A) and in KBC with brindle coat color (B) are shown. Black spots represents 167 expressed genes that are listed as coat color related genes in Gene card database and red spots represents 23 expressed genes/probes that are listed as pigment and melanocyte related genes in database of Gene ontology_bovine as well as coat color related genes in Gene card database. Spots located outside the two diagonal red and blue lines indicate more than 2-fold gene expression differences. Some spots were placed on top of each other depicting less than the total number.

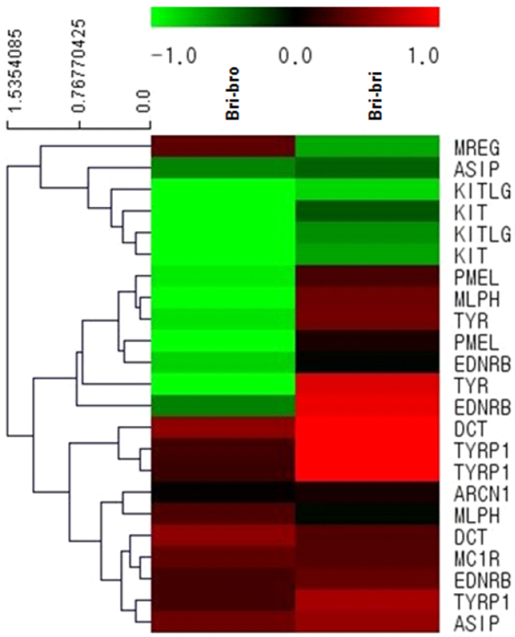


Fig. 4. Hierarchical clustering analysis of experimental groups and 23 selected genes/probes. Hierarchical clustering analysis display the expression pattern of 23 selected genes/probes before and after coat color appearance between Korean brindle cattle (KBC) with brown coat color (Bri-bro) and KBC with brindle coat color (Bri-bri). The red color indicates the up-regulated genes and green color indicates the down-regulated genes.

and brindle color. In the second cluster, there were PMEL, MLPH, EDNRB and TYR genes in a sequential manner. Their gene expression was low in KBC calves with brown coat color and relatively high in KBC calves with brindle coat color. In the third cluster, there were DCT and TYRP1 genes. Their gene expression was relatively high in KBC calves with brown coat color and high in KBC calves with brindle coat color. In the fourth cluster with smaller nested clusters, there were ARCN1, MLPH, DCT, MC1R, EDNRB, TYRP1, and ASIP genes. Their gene expression was relatively high in both KBC calves with brown and brindle coat color (Fig. 4).

DISCUSSION

In this study, the gene expression patterns of mature Korean brindle cattle (KBC) with different coat colors and Hanwoo cows were investigated by microarray and hierarchical clustering analysis. Gene expression patterns of KBC calves before

and after coat color appearance were also studied. Analysis of microarray data revealed that there were 5813 expressed genes/probes that were related to coat color, pigmentation, melanocyte, and melanogenesis. Among them, 23 genes/probes were listed both 1) as coat color associated genes in Gene card database and 2) as bovine pigment and melanocyte genes in Gene ontology_bovine database. Their expressions were further analyzed in detail.

KBC with brindle coat color expressed significantly higher (p<0.01) levels of ASIP, EDA, MLPH, PMEL, SLC45A2, TYR and TYRP1 genes than Hanwoo, while KBC with brindle coat color expressed significantly higher levels of CITED1, MLPH, NF1, PMEL, TYR and TYRP1 genes than KBC with brown coat color (Fig. 1). Thus, MLPH, PMEL, TYR and TYRP1 genes were commonly expressed in KBC with brindle coat color, whether they were compared with Hanwoo or KBC with brown coat color. Expression of these genes may be required for brindle coat color appearance in KBC. TYRP1 expression was 22.96 or 19.89 fold higher (p<0.01) in KBC with brindle color than either Hanwoo or KBC with brown color, respectively (Fig. 1). The data suggest that TYRP1 may be one of the genes contributing to brindle coat color in KBC, as TYRP1 synthesizes eumelanin (Berryers *et al.*, 2003). KBC with brindle coat color expressed higher levels of ASIP, EDA and SLC45A2 than Hanwoo, in addition to the MLPH, PMEL, TYR and TYRP1 genes. KBC with brown coat color expressed higher levels of ASIP, DCT, LEF1 and SLC45A2 genes than Hanwoo. Whether KBC has brindle or brown color, they expressed higher levels of ASIP and SLC45A2 genes than Hanwoo. This indicates that certain coat color associated genes, including ASIP and SLC45A2 are expressed higher in KBC with either brindle or brown coat color than in Hanwoo. Higher level of ASIP expression in KBC with brindle or brown color than in Hanwoo was unpredicted. Since ASIP is the natural antagonist of MC1R (Barsh, 1996), lower expression of ASIP in KBC than in Hanwoo would have been expected. SLC45A2 is solute carrier family 45, member 2, also known as melanoma antigen AIM1 (Fukamachi *et al.*, 2001). It may be involved in the melanogenesis of cattle hair cells. The function of SLC45A2 needs to be investigated further in the coat color appearance of KBC. Since KBC with brown coat color have capacity to express certain coat color associated genes at higher level than Hanwoo, KBC with brown coat color may have genetic potential to express brindle coat color in their offspring when they are bred with appropriate KBC cattle.

Microarray analysis revealed that MC1R was one of the 23 selected genes/probes (Table 1) that were expressed and its expression was relatively high (Fig. 2). However, level of MC1R expression did not change more than 2 fold among experimental groups with different coat colors. The expression levels of MC1R in KBC with brown, brindle and black colors were 1.50, 1.65, and 1.87 fold higher (p<0.01) than Hanwoo, respectively. The results may be comparable to the previous study showing variation of MC1R expression in KBC with different coat colors (Lee *et al.*, 2014). Though the gene is known for most variation in coat color, the genotype of MC1R may be more critical (Park *et al.*, 2012) than the level of expression in the coat color appearance of KBC. PMEL was reported to interact with MC1R (Schmutz *et al.*, 2013) and its expression was relatively high (Fig. 2). Therefore, PMEL interaction with MC1R may require further investigation in the future.

Only a few genes expressed more than 2 fold in KBC calves with brindle coat color after brindle coat color appearance than before (Fig. 3B). Expression of TYRP1 gene was 2.18 fold higher (p=0.02) in KBC calves with brindle coat color after brindle coat color appearance than before. TYRP1 could be one of the genes that are expressed from the early stage of growth in KBC. The pattern of differential gene expression shown in the mature cattle with different coat colors may take place later as the calves grow. Changes of hormone levels, including steroid hormones, in KBC calves may also influence the coat color appearance during their growth and maturation (Lee *et al.*, 2015). Though it was not listed as one of the 23 selected genes/probes in Table 1, ZIC2 gene expression was 17.0 or 14.9 fold higher in KBC calves with brown color 1or brindle color, after brindle coat color appearance in KBC calves with brindle color than before. ZIC2 is zinc finger protein of cerebellum 2 and it is known to be expressed in the fetal brain and retina (Nagai *et al.*, 1997). The expression of ZIC2 needs to be confirmed in the hair cells of KBC calves by other methods. Since its expression was elevated similarly in both the KBC calves with brown and brindle color, it may not affect the coat color significantly. While EDNRB expression was relatively low in mature experimental animal group contrasts (Fig. 2), it was relatively high in KBC calves, with higher expression in KBC calves with brindle color than brown color (Fig. 4). EDNRB has been implicated in the black color trait in a Chinese pig breed (Wang *et al.*, 2015) and may have a

potential to influence coat color in KBC.

In conclusion, differential gene expression pattern was determined in mature KBC with different coat colors and in Hanwoo cows. Out of many expressed genes, 23 selected genes/probes were 1) coat color associated genes listed in the Gene card database and 2) pigment and melanocyte genes listed in the Gene ontology_bovine database. Among the 23 genes/probes, MLPH, PMEL, TYR and TYRP1 genes were expressed at least two fold higher ($p<0.01$) levels in KBC with brindle color than either Hanwoo or KBC with brown color. TYRP1 gene expression showed the biggest fold difference. There were a few novel genes that were expressed, including SLC45A2 and ZIC2. Results of the differential gene expression among KBC with different coat colors and in Hanwoo may be used to complement the current breeding scheme, which is mainly based on the MC1R genotypes and coat color, to improve coat color appearance in KBC. Expression pattern of coat color associated genes that were determined from this study may be considered in the breeding scheme of KBC. Confirmation of novel or unpredicted gene expression, including LEF1, EDA, SLC45A2, SOX10, CITED1, NF1, LOC785371 and ZIC2 by additional methods may be needed. Studies on the regulation and mechanism of gene expression of highly expressed genes would be next steps to better understand coat color determination and to improve brindle coat color appearance in KBC.

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Received May 6, 2015, Revised June 12, 2015, Accepted June 14, 2015