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Changes in Melanogenesis-Related Genes by the Variation of MC1R in Dark-Muzzled Korean Native Cattle and Korean Brindle Cattle

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Abstract: Researchers observed that the MC1R genotype of Korean native cattle with dark muzzle was e/e or E+/e while the genotype of Korean native cattle with light muzzle was E+/E+ which is a variant of the MC1R genotype in the Korean native cattle. However, 2 genetic variations, E+/e with 4 bands of 174, 207, 328 and 535 bp and E+/e with 3 bands of 174, 207 and 328 bp were identified in Korean brindle cattle. The dark muzzle in Korean native cattle is formed by eumelanin production through MC1R expression which was higher in the melanocyte region of the tissue in Korean native cattle with light muzzle and Korean brindle cattle. However, in Korean brindle cattle while MC1R expression in the melanocytes of tissues and in total mRNA was not higher than in Korean native cattle with dark muzzle, β-catenin formation which affects MITF production was high. High expression of TYR and DCT, the 2 most important factors to increase eumelanin synthesis, increased dark muzzle formation.

Key words: Melanogenesis gene, MC1R, muzzle, cattle, Korean brindle cattle

INTRODUCTION

Changes in the mechanism of melanogenesis are very important in the determination of coat color in mammals. MC1R (Melanocortin 1 Receptor) is one of the most importantdetermining factors that cause the influx of Melanocyte Stimulating Hormone (α-MSH) into melanocytes (Suzuki *et al*., 1996; Seo *et al*., 2007). The other important factors are the variation in MC1R phaomelanin which forms red or yellow color and eumelanin which forms black or brown coat color, both of which are important genes that determine coat color of mammals (Jackson, 1993; Robbins *et al*., 1993). Recently, it was reported that during the process of melanin biosynthesis, the antagonistic action of Agouti factors that block the coupling of MC1R and α-MSH can stimulate phaomelanin synthesis (Barsh, 1996; Rieder *et al*., 2001) and expression of Agouti factors working with the expression or variation of MC1R is very important for the determination of coat color (Girardot *et al*., 2005, 2006). Melanogenesis in mammals is determined by the activation of various mechanisms and with the MC1R mechanism occurring in the cell membrane of melanocytes being the core mechanism (Slominski *et al*., 2004; Selz *et al*., 2007). By the MC1R mechanism, α-MSH enters the cytoplasm and is coupled with the Gs subunit of guanine nucleotide-binding protein after which Adenyl Cyclase (AC) is activated (Park and Gilchrest, 1999; Saito *et al*., 2003). As a result of the cAMP (cathelicidin antimicrobial peptide) increase inside the melanocyte, Protein Kinase A (PKA) is activated and it increases the biosynthesis of Cyclic Amp-Responsive Element-Binding protein (CREB) and Creb-Binding Protein (CBP) thereby increasing the production of Microphthalmia-Associated Transcription Factor (MITF) in DNA (Deng *et al*., 2008, 2009; Zhang *et al*., 2010). By the Extracellular Signal-Regulated Kinase 1/2 (ERK1/2) derived from Mitogen Activated Protein Kinase (MAPK) signaling, MITF is activated and coupled with the M box located in the promoter region of genes thereby activating transcription of the target genes of Tyrosine Metabolism (TYR), Tyrosinase-Related Protein 1 (TYRP1) and Dopachrome Tautomerase (DCT) (Shen *et al*., 2012; Zhou *et al*., 2012). It is known that MITF induces epistasis to control the formation of the melanosome complex. The other mechanism of melanogenesis is the stimulation of MITF production by Wnt signaling. Wnt signaling is very important in the determination of cell fate, proliferation and differentiation (Kang *et al*., 2011). In particular, it helps the stabilized expression of β-catenin which promotes gene expression and Transforms Transcription Factor 7 (TCF7) and Lymphoid Enhancer-binding Factor 1 (LEF1) into transcription activation factors thereby increasing the MITF

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production in melanocytes (Kawano and Kypta, 2003; Kim *et al*., 1995; Gunathilake *et al*., 2009; Kang *et al*., 2011). However, since the role of Wnt signaling is very broad, it may not be directly involved in the production of MITF. Therefore, research on coat color determination has been focused on the genetic variation of MC1R and Agouti. However, Han *et al*. (2011) speculated that the genetic variation of Agouti had no effects on the development of coat color in Korean native cattle and their crossbred progeny and did not play an important role in the variation of coat color. So, the variation of MC1R is important for the coat color variation. However, there are only a few reports on the effects of MC1R variation on the genes related to melanogenesis in muzzle color variation.

This experiment was conducted to investigate the roles of MC1R genotype in the pigmentation changes of muzzle color and coat color through the analysis of the expression patterns of genes related to melanogenesis in the muzzle color variation in Korean brindle cattle and Korean native cattle with dark or light muzzle color to obtain basic information on the control of color variation and to increase the brindle pattern in Korean brindle cattle.

MATERIALS AND METHODS

Experimental samples: The muzzles of Korean native cattle used in this experiment were collected from Pyungnong slaughterhouse located in Pyungtaeck, Kyeonggi Province. They were classified into light, very dark, medium dark and weak dark (Lee *et al*., 2011). Eighteen heads of cattle with medium dark muzzle and 18 heads of cattle without dark muzzle were selected. Of Korean brindle cattle raised in Ulreundo Agricultural Technology Center, cattle with very dark muzzle and with coat color of black (70%) and yellow (30%) were selected and used for the experiment (Fig. 1). For the control, dairy (Holstein) cattle slaughtered at Pyungnong slaughterhouse were used. Purebred Korean native cattle certified by Rural Development Administration (RDA) were selected and the fore parts of muzzles were cut by surgical incision and stored in a liquid nitrogen tank (-196°C) and transported to the laboratory (Table 1).

DNA extraction and MC1R pattern analysis: Muzzles were crushed in liquid nitrogen (-196°C) and DNA was separated using A DNA extraction kit (Toyobo, JAN) and dissolved in sterilized water using magnetic beads. Sequences of MC1R primers for PCR-RFLP of MC1R were taken from NCBI (http://www.ncbi.nlm.nih.gov, Gene accession No. AF445642) (Klungland *et al*., 1995; Chung *et al*., 2000) (Table 1). The purity of the DNA was ascertained by using A Nano-spectrometer. DNA of A_{260}/A_{280} 1.8~1.9 was selected and DNA concentration was made up to 200±30 ng μL^{-1}. The PCR reaction mix consisted of 10 pmol of forward/reverse primer, 1 μL of 2.5 mM DNA, 2.5 μL of 10X PCR buffer, 2 units of Taq polymerase (Toyobo, JPN) and made up to 17.5 μL using distilled water. The reaction mixture was pre-denatured at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min. To analyze the genetic variation of *MC1R* gene, 10 μL of PCR product was mixed with 2 units of MspI (Toyobo, JPN) and 2 μL of 10X M buffer at 37°C for 4 h. After enzyme treatment, electrophoresis was performed using 2% agarose gel and DNA bands were identified and classified by the methods of Klungland *et al*. (1995) and Park *et al*. (2012).

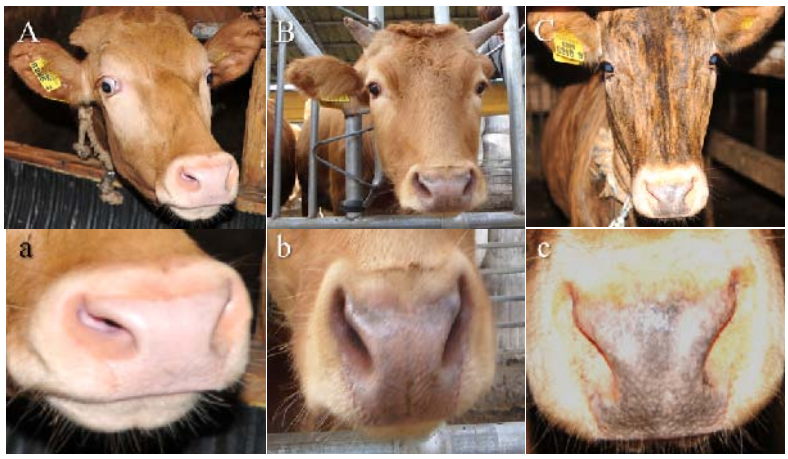

Fig. 1: Criteria for the classification of the muzzle and coat color of Korean brindle cattle; A, B) Yellow coat color; C) Brindle coat color; a) White muzzle; b, c) Dark muzzle

Table 1: Primers for real-time RT-PCR and PCR-RFLP analysis of MC1R genotype and melanogenesis-associated genes

Primer symbol	Sequence (mRNA)	Size	Primer symbol	Sequence (mRNA)	Size
MC1R	Fw 5' GTGCCTGGAGGTGTCCATC 3'	300	MITF	Fw 5' 5' AAGCCTCCGTTAAGCTCCTC 3'	140
	Rv 5' AAGCAGAGGCTGGACACCAT 3'			Rv 5' GCCACTCTCTGTTGCATGAA 3'	
GNAs	Fw 5' TGTCTCGGAAACAGCAAGACC 3'	203	DCT	Fw 5' AGATGGGACCCTGGACTCTC 3'	141
	Rv 5' CCGGTAGACCTGCTTGTCCT3'			Rv 5' GGACAACCAAAACCACGAGC 3'	
cAMP	Fw TGTTATGGCGTCTTCCCCAG 3'	200	CTNNB1	Fw 5' ATTTGATGGAGTTGGACATGGC 3'	226
	Rv 5' TTCAAGCACTGCCACTCTGT 3'			Rv 5' CCAGCTACTTGTTCTTGAGTGAAGG 3'	
TYRP1	Fw 5' AACACCTGCGACATTTGCAC 3'	176	TYR	Fw 5' TAACAGAACCTGCCAGTGC 3'	90
	Rv 5' GTCCCCCTGTTCCATTCAGG 3'			Rv 5' CTTTCTGTGCAGCGGGGTCCCCTAGA 3'	
PRKCB	Fw 5' GCTTCCCTTTGGTTCGTCATTC 3'	205	CREB1	Fw 5' GCAAAGTGGCCAAACCTGACTA 3'	269
	Rv 5' CCTCAAGCAACTGACCAACACA 3'			Rv 5' TGAACTCAGAGAAGACGGAGCA 3'	

Gene name GeneBank accession No. = MC1R (AF445642); Sequence (DNA) = Fw 5' CAGTGCCTGGAGGTGTCCAT 3', Rv 5' GGCCAGCATGTGGACGTAGA 3'; Size = 535 bp; Enzyme = Msp1

Table 2: Coat and muzzle colors of Korean cattle

		Coat color					
		Brindle			Nose		
Sex	Symbol of sample	Non[a]	Part-whole[b]	Whole[c]	Yellow	Black dot	Total
Male	KNC	12	-	-	12	-	12
	KNCDM	12	-	-	-	12	12
	KBC	-	4	8	-	12	12

KNC = Korean Native Cattle (Han Woo); KNCDM = Korean Native Cattle with Dark Muzzle; KBC = Korean Brindle Cattle; [a]Non-whole body is yellow; [b]Part-whole: 10~50% of the whole body; Whole: ≥50% of the whole body

RNA extraction: The total RNA was extracted by Trizol Method (Invitrogen, CA, USA). RNA of purity within a range of 1.7~1.8 was used. cDNA for real time RT-PCR was synthesized using 1 μg of RNA by the 1st strand synthesis method using oligo primers (Oligo dT primer, Invitrogen, CA, USA). They were quantified using the bovine Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) mRNA housekeeping gene.

Real time RT-PCR: After identification of MC1R variation patterns, Korean native cattle with light muzzle (E+/E+), Korean native cattle with dark muzzle, Holstein (ED/ED) and Korean brindle cattle with 4 band pattern (E+/e) were selected (8 heads in each group) and primers for the genes responsible for melanogenesis and which play an important role in melanin biosynthesis were prepared using base pair sequences of genes registered in NCBI GeneBank (Table 2). In each group, 1 μL of cDNA was used as a mold and one step SYBR RT-PCR kit (TaKaRa, Shiga, Japan). The 20 pmol of primer (forward, reverse) of each DNA was mixed and the total volume was made up to 25 μL using distilled water. For the analysis, Line-gene K (Bioneer Technology, Tokyo, JPN) real time RT-PCR was repeated >3 times. After real time RT-PCR using Rotor Gene Real Time Software 6.0 Cycle threshold value (Ct) was converted to fold change value based on the semi-log amplification plot of geometric region.

***In situ* hybridization of MC1R mRNA:** RNA primers were prepared based on the mRNA sequence of the MC1R exonic regions that are the sites of genetic variation and the cDNA of the dairy cattle was amplified using RT-PCR. A 300 bp band of wild type MC1R whose region of genetic variation was identified by MspI (Joerg *et al*., 1996) was extracted using a Gel extraction kit (Toyobo, JPN). A digoxigenin-labeled hybridization kit (ROCHE, Mannheim, GER) probe was prepared according to the manufacturer's protocol. Tissues with epidermal and dermoepidermal layers containing melanocytes in the muzzle tissues of cattle were fixed in 70% ethanol with 0.2% Diethyl Pyrocarbonate (DEPC) for 24 h and dehydrated sequentially. Paraffin was used to prepare tissue block which was cut into sections 10 μm thick and the slides were prepared. Then, paraffin was removed and the labeled probe was added to RiboHybe hybridization solution (Toyobo, Osaka, JPN). The *in situ* hybridization analysis was performed at 65°C for 16 h. After *in situ* hybridization, samples were re-fixed with 0.2X SSC containing 60% formamide and the antibody reaction was induced using anti-digoxigenin antibody at 37°C for 2 h. Then, NBT/BCIP stock solution (0.18 mg mL^{-1} BCIP, 0.34 mg mL^{-1} NBT and 240 μg mL^{-1} levamisole) was added to the slide and the slides were incubated for 2 h in dark for color development. The methyl green-stained nucleus was observed under optical microscope.

Statistical analysis: Data were subjected to a t-test and GLM of the Statistical Analysis System (SAS Institute, Version 9.4, Cary, NC, USA). The analyzed values are shown as mean±SD and $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Genetic variation of MC1R in Korean native cattle with dark or light muzzle and Korean brindle cattle: Using the DNA extracted from muzzles, the genetic variation of MC1R was analyzed. The genotype of Korean native cattle with dark muzzle was e/e or E+/e while the genotype of Korean native cattle with light muzzle was E+/E+ which was a variation of MC1R in Korean native cattle.

However, 2 genetic variations, E+/e with 4 bands of 174, 207, 328 and 535 bp and E+/e with 3 bands of 174, 207 and 328 bp were identified in Korean brindle cattle (Fig. 2a-c).

Location and patterns of MC1R mRNA expression: MC1R mRNA was expressed in melanocytes of all epidermal cells of muzzles in all cattle. However, the expression rate was lower in Korean native cattle with light muzzle than in Korean native cattle with dark muzzle, Korean brindle cattle and dairy cattle. The mRNA of MC1R was not expressed in the dermoepidermal layer of Korean native cattle with light muzzle. In dark-muzzled Korean native cattle, the mRNA was expressed in the melanocytes of the stratum layer which connects the epidermal and dermoepidermal layers and was highly expressed in the stratum spinosum and granulosa wall. In Korean brindle cattle and dark-muzzled Korean native cattle, a similar expression pattern was noted but MC1R expression was low in the melanocytes of the stratum layer. A similar expression rate was noted in the Langerhans cells of Korean native cattle with dark muzzle and Korean brindle cattle. In all muzzles of dairy cattle, mRNA of MC1R was highly expressed especially in all tissues containing melanocytes (Fig. 3).

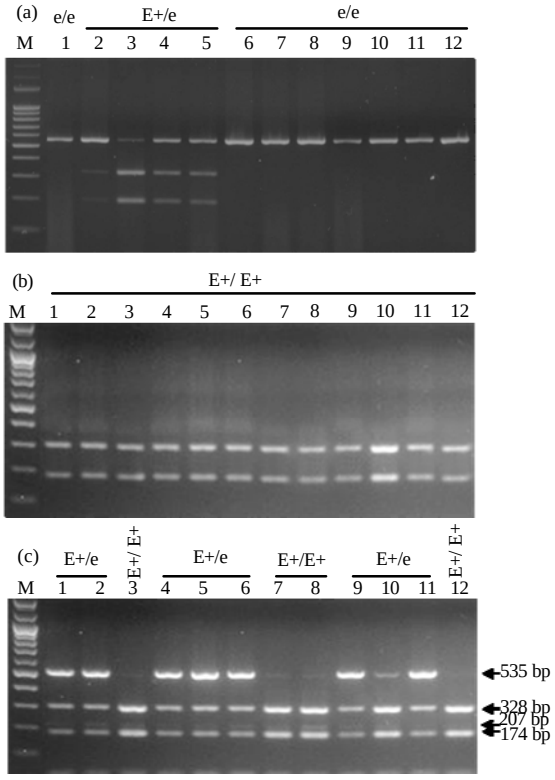

Fig. 2: Genotype mutation of the *MC1R* gene in Korean native cattle, Korean native cattle with dark muzzle and Korean brindle cattle. PCR-RFLP analysis for detection of the MC1R genotype mutation in cattle. a) Korean native cattle; b) Korean native cattle with dark muzzle; c) Korean brindle cattle

Expression patterns of melanogenesis-related genes in muzzles: The expression rate of melanin biosynthesis-related genes was analyzed using real time RT-PCR (Fig. 4). The expression of Agouti factor, ASIP which inhibits MC1R was more highly expressed in Korean native cattle with light muzzle and Korean brindle cattle than in Korean native cattle with dark muzzle and dairy cattle while the expression of MC1R was relatively low. However, GNAS (Guanine Nucleotide-binding protein G (s) subunit alpha) which activates AC factor when coupled with MC1R was highly expressed in Korean native cattle with light or dark muzzle and it was significantly low in Korean brindle cattle and dairy cattle. Also, PRKCB (Protein Kinase C, B) which stimulates CREB/CBP coupling was expressed at a significantly lower level in Korean native cattle with light muzzle and Korean brindle cattle but highly expressed in dark-muzzled Korean native cattle and dairy cattle. Also, MITF and CREB1 which activate MITF production were highly expressed in all groups of cattle except Korean native cattle with light muzzle. However, CTNNB1 (catenin (cadherin-associated protein), beta 1) which codes for β-catenin that stimulates MITF production in DNA by increasing TCF/LEF 1 coupling was expressed at a significantly higher level in Korean native cattle and Korean brindle cattle while its expression was low in Korean native cattle with dark muzzle and dairy cattle. The expression of TYR and DCT which directly affect eumelanin or phaomelanin production in the melanosome was very low in Korean native cattle and TYR was highly expressed in Korean native cattle with dark muzzle while DCT was highly expressed in Korean native cattle with dark muzzle and Korean brindle cattle. However, TYRP1 expression was significantly high in Korean native cattle and dairy cattle and was lowest in Korean brindle cattle.

The genetic variation of MC1R and Agouti plays an important role in the formation of different coat colors and in the fixation of brindle coat color in Korean brindle cattle (Barsh, 1996; Rieder *et al*., 2001). Although, genetic variation of stock animals is used to fix coat color of the progeny, coat color expression and muzzle color variation cannot be explained only by the genotypes of MC1R and Agouti factors (Han *et al*., 2011). Although, it was reported that there was a linkage between coat color change and muzzle color difference (Lee *et al*., 2011; Park *et al*., 2012) little has been reported about the

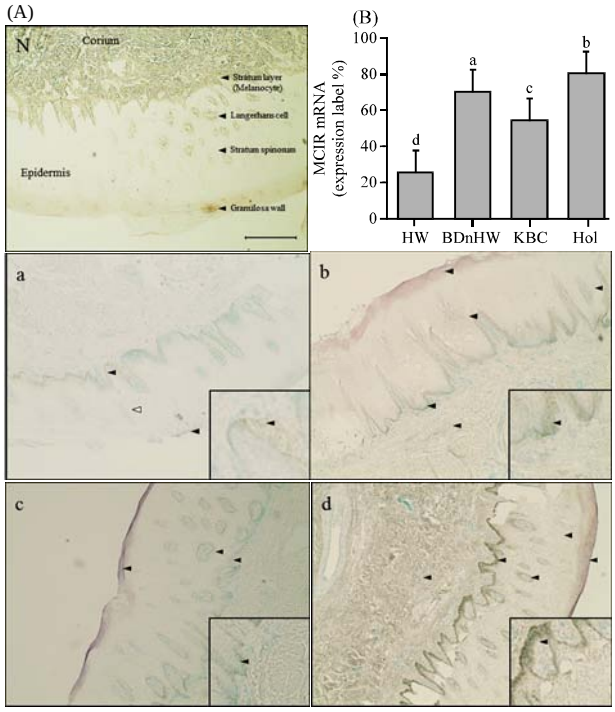

Fig. 3: *In situ* hybridization of MAP1LC3A mRNA in the muzzle tissue of cattle. Pre-hybridization solution was used as a control for the Holstein cattle below the negative panel. Scale bar = 100 μm. Black arrows indicate MC1R RNA probe detection. a-f) 100x magnification; a-1-f-1: 200x magnification). A) *In situ* hybridization analysis of the *MC1R* gene in the cattle muzzle; B) Percentage of MC1R probe detection dots. ***Different letters within the same column represent a significant difference ($p<0.05$); a) Korean native cattle; b) Korean native cattle with dark muzzle; c) Korean brindle cattle; d) Holstein cattle

expression patterns of genes related to melanin biosynthesis in melanocytes through the MC1R variation in genes related to muzzle color variation (Park and Gilchrest, 1999; Saito *et al*., 2003). Variation in MC1R has significant effects on the melanogenesis by α-MSH (Slominski *et al*., 2004; Selz *et al*., 2007) and this variation affects the expression of coat color.

In this experiment, MC1R variation in Korean brindle cattle and Korean native cattle with dark or light muzzle and dairy cattle were studied and changes in genes related to melanogenesis were analyzed.

The genotype of light-muzzled Korean native cattle was E+/E+ with the main allele E+ which is similar to the result by Son *et al*. (2000). Many Korean native cattle with dark muzzle whose pedigree was certified by RDA had genotype of e/e with the basic allele e than the genotype of E+/e with the basic allele E+. However, the genotype of Korean brindle cattle was E+/E+ with 4 bands of genetic variation or E+/e with 3 bands which was similar to the result of Park *et al*. (2012) and when compared to Korean native cattle, the different variation of MC1R was detected by the band formed at 207 bp. Generally, coat color is determined by the distribution of eumelanin (black/brown) and pheomelanin (yellow/red) and its expression is mainly controlled by the Extension (*E*) gene (Jackson, 1993; Robbins *et al*., 1993; Royo *et al*., 2005). In the various cattle breeds, the dominant black coat color is expressed by dominant genotype of ED, red or brown is expressed in the frame shift mutation of recessive homozygous e by base pair deletion and the wild-type of E+ is expressed in various coat colors (Klungland *et al*., 1995; Lee *et al*., 2011; Rouzaud *et al*., 2000). However, in this experiment, a novel MC1R variation was observed in Korean brindle cattle as a new restriction site cut in the *MC1R* gene of Korean native cattle with or without dark muzzle and Korean brindle cattle. This indicates that as a result of MC1R variation, a different biosynthetic mechanism of melanin may be present during melanogenesis. The final goal of melanogenesis in melanocytes is to produce MITF which increases gene production by binding to the M-binding region located in the DNA promoter region of TYR, DCT and TYRP1 (Bennett and Lamoreux, 2003; Berryere *et al*., 2003; Guibert *et al*., 2004; Chen *et al*., 2013) and is controlled by the epistatic action of MC1R

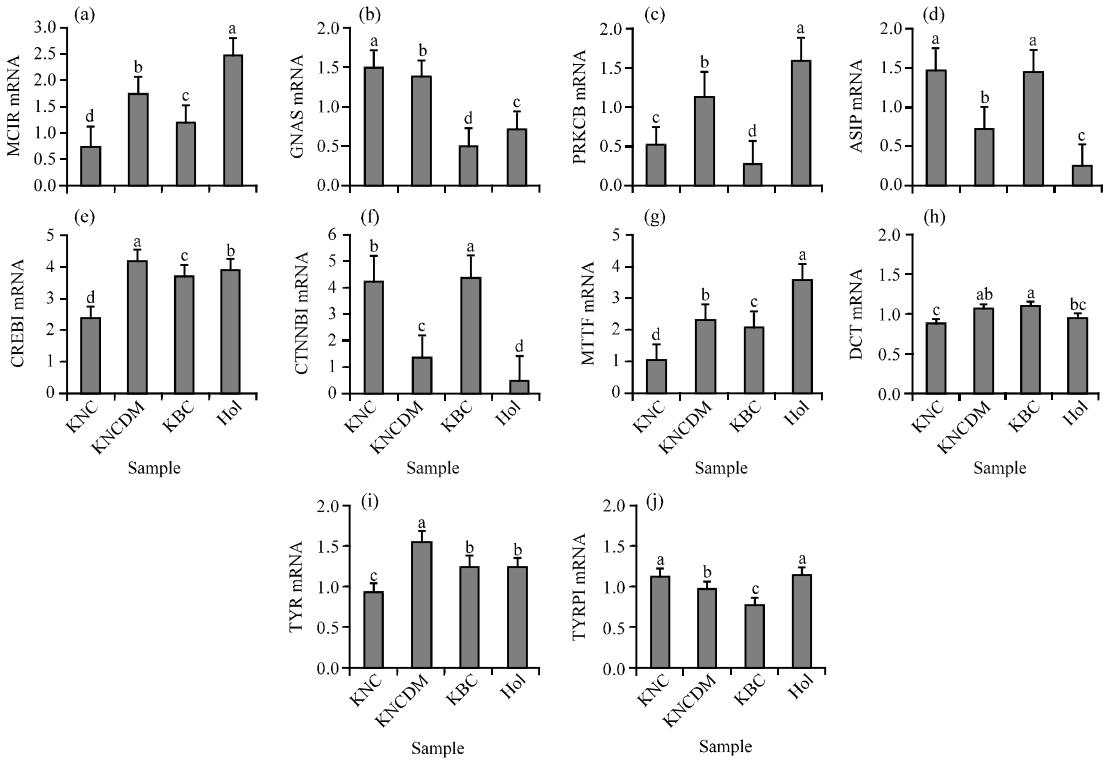

Fig. 4: a-j) Expression patterns using real-time RT-PCR analysis of melanoma-associated genes between the muzzle tissue mRNAs in KNC, KNCDM, KBC and Hol. [a-d]Different letters within the same column represent a significant difference ($p<0.05$). KNC: Korean Native Cattle (Han Woo); KNCDM: Korean Native Cattle with Dark Muzzle; KBC: Korean Brindle Cattle; Hol: Holstein cattle

expression which leads to α-MSH synthesis while ASIP inhibits α-MSH synthesis (Girardot *et al*., 2006). In dark-muzzled Korean native cattle having the e/e genotype, the expression of ASIP which inhibits MC1R was higher than in light-muzzled Korean native cattle with the E+/E+ genotype. In Korean brindle cattle with E+/e genotype having 4 bands, the expression of MC1R increased, indicating an interaction at the mRNA level. The expression of GNAS which binds to MC1R in melanocyte (Saito *et al*., 2003) was higher in Korean native cattle with light or dark muzzle than in Korean brindle cattle and dairy cattle. Also, PRKCB (PKA) which is involved in CREB/CBP synthesis that plays an important role in MITF synthesis (Busca and Ballotti, 2000) was highly expressed in Korean native cattle with dark muzzle and in dairy cattle while CREB and MITF expression was low in light-muzzled Korean native cattle. However, in Korean brindle cattle, the expression of CREB and MITF was similar to that of Korean native cattle with dark muzzle regardless of PKA expression. Expression of the *β-catenin* gene CTNNB1 that is involved in MC1R synthesis in melanocytes by different signaling (Saito *et al*., 2003; Chen *et al*., 2013) was significantly higher in Korean native cattle with light muzzle and Korean brindle cattle. In Korean brindle cattle, MITF production through the MC1R mechanism and MITF production by β-catenin which is activated by Wnt signaling, seems to be activated at the same time and their activity seems to increase through binding of the M-region of promoters of TYR, TYRP1 and DCT with the produced MITF. Since, the expression of TYR and DCT which plays an important role in phaomelanin or eumelanin synthesis through melanin biosynthesis was high in Korean native cattle with dark muzzle and Korean brindle cattle their eumelanin production may increase (Berryere *et al*., 2003; Guibert *et al*., 2004). However, expression of TYRP1 which is involved in melanin biosynthesis (Berryere *et al*., 2003) was high in Korean native cattle with light or dark muzzle while it was low in Korean brindle cattle. TYRP1 is known to increase eumelanin production by coupling with DHICA (5,6-dihydroxyindole-2-carboxylic acid oxidase) synthesized by DCT (Olivares *et al*., 2001) and was highly expressed in Korean native cattle with dark muzzle with low expression in Korean brindle cattle. Eumelanin synthesis mechanism may be different in the muzzle of

Korean native cattle and in the brindle coat color of Korean brindle cattle due to the different expression patterns of genes involved in melanin biosynthesis. In Korean native cattle, the dark muzzle is formed by eumelanin production through MC1R expression and the expression in the melanocyte region of the tissue was higher in dark-muzzled Korean native cattle than in Korean native cattle with light muzzle and Korean brindle cattle. However, in Korean brindle cattle, MC1R expression in the melanocytes of tissues and in total mRNA was not higher than in Korean native cattle with dark muzzle but β-catenin formation which affects MITF production was high. High expression of TYR and DCT which are the two most important factors to increase eumelanin synthesis may increase dark muzzle formation. Therefore, the melanogenesis pathway for the formation of dark muzzle in Korean native cattle and the brindle pattern in Korean brindle cattle seems to be different due to the different formation patterns of MC1R variation.

CONCLUSION

Therefore, the results suggest that control of melanogenesis in the formation of dark muzzle in Korean native and brindle cattle is different due to the different patterns of mutations in the MC1R genotype.

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