

Research Article



Differential Methylation of Melanin-related Epigenetic Genes during Brindle Cattle Growth

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Abstract | Coat pigmentation in mammals is determined by the relative distribution of pheomelanin and eumelanin. The phenotype of these two pigments, when expressed in melanocytes, is regulated by the mechanism of melanogenesis and the agouti locus alleles. Therefore, the present study analyzed mutations in the coat color-related genes in two Korean cattle breeds i.e., Hanwoo and Korean brindle cattle. According to results the coat color-related epigenetic markers [differentially methylated CpGs (DM CpGs)], by reduced representation bisulfite sequencing showed that brindle cattle has the biological functions of DM CpG-associated genes. In particular, the present study analyzed the genetic variation of the post-forming factor and expression patterns of the genes that affect the coat color of brindle cattle and revealed that the determination of coat color of brindle cattle was distinct from that of other Korean Hanwoo. Thus, the mutation or activity of major factors of MAPK were involved in melanogenesis by the activation of melanocortin 1 receptor (MC1R). In addition, the MARK mechanism significantly affected melanogenesis, even at lower concentrations of MC1R. These results suggested the possibility that hair color could be determined by the mutation of the agouti gene in the black coat color section. Therefore, the results of this study could help in the modulation of coat color and positive selection of the Korean brindle cattle.

Keywords | Brindle cattle, Epigenetic genes, Melanin, Melanocytes, Methylation

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INTRODUCTION

The history of the nation's cattle began about 4,000 years ago with the hybridization of Indian zebu (*Bos zebu*) and European cattle (*Bos taurus*), and it has been reported that this hybrid was carried to Korea by the Chinese, Mongolians, and Manchurians. Kim et al. (2000) could not confirm the exact basis of the history and origin due to difficulties in securing samples. An important factor in determining the breed of livestock is the coat color. The change in the mechanism of melanogenesis, in turn, is very important in determining the coat color

of mammals. Melanocortin 1 receptor (MC1R), which is activated by the introduction of melanocyte stimulating hormones (α -MSHs) in melanocytes, is a typical coat color determinant (Seo et al., 2007; Goud et al., 2019). It forms pheomelanin, which denatures red or yellow color through genetic mutation, and eumelanin, which is considered important in determining the coat color of mammals (Jackson, 1993; Robbins et al., 1993). It was reported that the offset action of the agouti factor in melanogenesis could interfere with the binding of α -MSHs to MC1R (Rieder et al., 2000; Choudhary et al., 2020), facilitating the production of pheomelanin. However, currently it

is known that the expression of the agouti factor in the determination of coat color plays an important role in activating the expression or variation of MC1R (Girardot et al., 2005, 2006). Microphthalmia-associated transcription factor (MITF) activates transcription of the target genes of tyrosine (TYR), tyrosinase-related protein 1 (TYRP1), and dopachrome tautomerase (DCT) metabolism (Zhou et al., 2012; Shen et al., 2012). In other words, MITF is recognized as a higher gene in the control of the formation of complex melanosomes. MITF is produced and facilitated by Wnt signaling, which is known to play a crucial role in controlling cell fate, proliferation, and differentiation (Kang et al., 2011). However, according to Han et al. (2011), the genetic variation of the agouti gene is assumed to have no effect on the development of a Korean Hanwoo or hybrid offspring, and it does not play a role in coat color variation. Meanwhile, Brown (2012) reported that the typical black/brown coat color gradually changed to yellow in castrated bulls of South China. Lee et al. (2015) reported that E2 concentration in expressed yellow to brindle colors increased before the determination of the initial coat color, but gradually decreased after the brindle color was formed. Bennett et al. (2003) was involved in the study of the control of pigments by MSH and melanocortin receptor (MCR) and regulation of cortisol, which is related to the adrenocorticotropic hormone (ACTH) and MC1R. In addition, POH (pro-opiomelanocortin) derivatives MSH and ACTH were regulated by receptors (including cortisol) of pigments, hormones, and steroid hormones. Hirobe et al. (2011) reported that E2 injection in mice increased the coat pigmentation. Therefore, the present study analyzed the genetic variation of the post-forming factor and expression patterns of the genes that affect the coat color of brindle cattle and revealed that the determination of coat color of Korean brindle cattle was distinct from that of other Korean Hanwoo.

MATERIALS AND METHODS

ETHICAL APPROVAL

All animal procedures followed the protocol approved by the Animal Experimentation Ethics Committee at Hankyong National University (permission number: 2018-2).

EXPERIMENTAL ANIMALS

Brindle cattle used in this experiment were bred by the National Institute of Animal Health in Chungcheongbuk-do and were classified as brindle, black, and yellow (Figure 1), according to the classification method of Park et al. (2016). To analyze the patterns of epigenetic expression of coat color during different growth stages, DNA of young brindle cattle (who reached the age of six months), whose coat color did not change after birth and began changing

at the age of less than one month, was extracted and used for analysis (Figure 2).

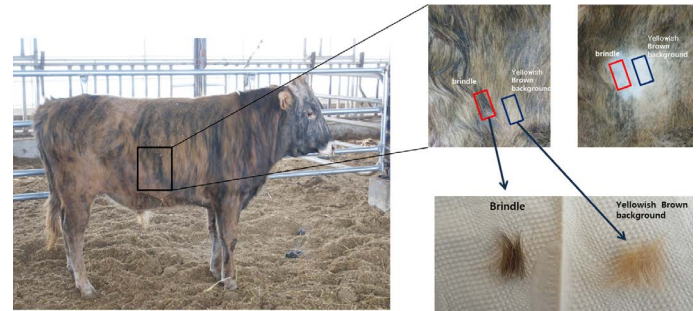


Figure 1: Samples for NGS analysis.



Figure 2: Samples for DNA methylation analysis- A: at 1 month of age, B: at 6 months of age.

NEXT-GENERATION SEQUENCING (NGS)

The NGS analysis was conducted using Bovine genome assembly UMD 3.1. It included information about the unknown genes and variation in the genome sequences expressed on chromosomes, and it did not apply to the repetitive genome sequences on the same line. The sequence of bases passed through the standard Illumina Chastity filter was analyzed further, and the sequence analysis was adjusted to a 90 base pair (bp) reading to reduce errors in the sequence in the last reading section of the first reading. Short-read mapping was completed using BBWA ver. 0.5.9. Data that could not be read were removed after mapping. To analyze the single-nucleotide polymorphisms (SNPs) and insertions and deletions (InDels), SAM tools and additional filters were used as follows:

1. SNPs and InDels with overall quality less than 20 were removed.
2. Variants with too low or too high read depth were removed. First, we calculated the mean and standard deviation (SD) read depth for all variants. Then we set the minimum as 10% of the mean and maximum as mean read depth + 3*SD read depth.
3. Variants with less than one forward or reverse alternative allele were removed.
4. Variants within five bp of each other were removed.
5. SNPs within five bp of an InDel were removed.
6. InDels within 10 bp of each other were removed.
7. Variants that have sites that were not in the reference genome were removed.

Following the SNP and InDel analyses, NGS-SNP (Grant et al., 2011) was used to allocate functional classes to each

SNP, and, where applicable, information on proteins and transcription proteins was provided. Functional analysis of the mechanism was performed using Ensembl release 68, Entrez Gene, NCBI, and UniProt (Flicek et al., 2011; Sayers et al., 2011).

REDUCED REPRESENTATION BISULFITE SEQUENCING (RRBS) LIBRARY PREPARATION METHOD

DNA (500 ng) was reacted with Msp1 (NEB) at 37°C for 24 h and then with ApeK1 (NEB) at 75°C for 20 h. Digested DNA was refined using the MiniElute PCR Purification Kit (Qiagen). It was then end-repaired with T4 DNA polymerase (NEB), klenow fragment (NEB), T4 polynucleotide kinase (NEB), dNTP, and 1x polynucleotide kinase buffer. The response was seen at 20°C after 30 min. Blunt ended DNA fragment with klenow (3'-5' exo-, NEB) was a-tailing at 37°C for 30 min. It was refined using the MiniElute PCR Purification Kit (Qiagen), and the adapter (illumina) was ligated using T4 DNA ligase (NEB) at 65°C for 15 min, after keeping it at 20°C for 15 min. DNA (160-420 bp) was selected using 2% agarose gel electrophoresis. According to the ZYMO EZ DNA manufacturer's instructions, bisulfite was converted using the methylation-Gold Kit (ZYMO). The bisulfite-converted products were PCR-amplified using PfuTurbo Cxhotstart DNA polymerase (Agilent technology). Primer used was as follows: forward: 5'-AATGATACGGCGACCACCGAGAT-3', reverse: 5'-CAAGCAGAAGACGGCATAACGA-3'. The PCR conditions were 11 to 15 cycles at 94°C for 10 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 5 min extension after 94°C for 1 min. The library was analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies) and quantified with real time PCR (RT-PCR).

STATISTICAL ANALYSIS

RT-PCR results were analyzed for statistical significance using the SAS package (Statistical Analysis System, Institute, version 9.4, Cary, NC, USA). The data are shown as mean ± SD, and the significant difference among the groups was determined at *p* value <0.05.

RESULTS

PATTERNS OF GENETIC EXPRESSION OF BLACK AND YELLOW HAIR SECTIONS IN BRINDLE CATTLE

NGS analysis of specific and non-specific expression genes in the black and yellow hair sections of the brindle cattle (brindle color) showed that the genes expressed in these two sections formed about 18,726 of the EST gene from about 1,000 bp to 10,000 bp (Figure 3). Among the differently expressed groups in the GO term of the gene, three processes of fourteen biological processes, seventeen cellular components, and seven molecular functions showed

significant differences. There was a significant difference (*p* <0.01) at the cellular component and biological process levels (cell cycle and activating factor). The results showed that the cell activating factors were different in the black and yellow sections of the brindle color. The number of genes expressed differently was high in the cellular component group in 321 biological processes, 702 cellular components, and 171 molecular functions, and a large number of melanosome intracellular non-membrane-bounded organelle related genes were found (Table 1). In this experiment, a comparison of the expression pattern of genes involved in melanogenesis, among the separated genes, confirmed that the expression of seven factors such as TYRP and MITF, which are related to melanocytes, in the black section was more than five times greater than that in the yellow zone. It was also confirmed that eumelanin of 49 major factors, such as MARK-related factors associated with melanogenesis, was involved (Figure 4). Thirty factors were found to be expressed more than five times in the black color section of brindle color, demonstrating that the mechanism and composition of melanogenesis in the yellow and black sections were different.

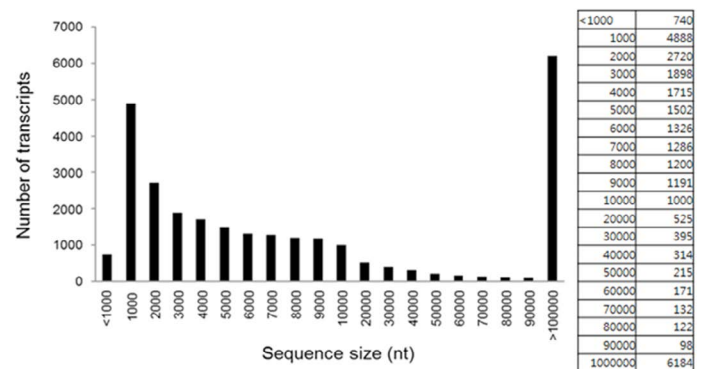


Figure 3: Length distribution and abundance of all transcripts identified in both the yellow background and brindle section.

THE CHANGE IN THE COAT COLOR ACROSS GROWTH STAGES

Analysis of the change in coat color at each growth stage predicted that 68% of 28 brindle cattle showed a change in coat color at about one to six months of age. Additionally, it was confirmed that 50% of 61 brindle cattle showed a change in coat color at about one to six months of age (Figure 5).

THE CHANGE IN DNA METHYLATION ACROSS GROWTH STAGES

Accordingly, the significant level of FDR <0.01 and methylation difference >25% was applied in the results of gene methylation analysis on DNA across growth stages of brindle cattle. About 1395 were selected for group comparisons (3 week vs. 6 month), and 1493 to 7551 DM CpGs were selected for individual comparisons.

Table 1: Top 30 highly expressed genes in the brindle section compared to the yellow background.

GO terms			Total number of genes	Template matching (P<0.01)
Biological process	GO:0007398	ectoderm development	17	6.05E-06
	GO:0008544	epidermis development	16	1.26E-05
	GO:0060429	epithelium development	18	7.04E-04
	GO:0060429	epithelium development	18	7.04E-04
	GO:0030216	keratinocyte differentiation	7	7.63E-04
	GO:0007264	small GTPase mediated signal transduction	28	0.001966
	GO:0009913	epidermal cell differentiation	7	0.002496
	GO:0018149	peptide cross-linking	7	0.002496
	GO:0022610	biological adhesion	43	0.002652
	GO:0007155	cell adhesion	43	0.002652
	GO:0006468	protein amino acid phosphorylation	58	0.003019
	GO:0030855	epithelial cell differentiation	11	0.004316
	GO:0051056	regulation of small GTPase mediated signal transduction	26	0.004546
	GO:0016337	cell-cell adhesion	22	0.007546
	Cellular component	GO:0005856	cytoskeleton	98
GO:0045111		intermediate filament cytoskeleton	30	1.41E-10
GO:0005882		intermediate filament	30	1.41E-10
GO:0045095		keratin filament	21	1.44E-09
GO:0005911		cell-cell junction	27	3.65E-08
GO:0043296		apical junction complex	19	3.57E-07
GO:0016327		apicolateral plasma membrane	19	4.85E-07
GO:0044430		cytoskeletal part	64	1.44E-06
GO:0044430		cytoskeletal part	64	1.44E-06
GO:0030054		cell junction	38	6.75E-06
GO:0005923		tight junction	13	1.13E-04
GO:0070160		occluding junction	13	1.13E-04
GO:0001533		cornified envelope	5	7.05E-04
GO:0043232		intracellular non-membrane-bounded organelle	125	0.002658
GO:0043228		non-membrane-bounded organelle	125	0.002658
GO:0030057	desmosome	5	0.004801	
GO:0005922	connexon complex	6	0.009066	
Molecular function	GO:0016755	transferase activity, transferring amino-acyl groups	9	1.87E-05
	GO:0016755	transferase activity, transferring amino-acyl groups	9	1.87E-05
	GO:0004713	protein tyrosine kinase activity	22	2.71E-04
	GO:0005198	structural molecule activity	56	5.87E-04
	GO:0004672	protein kinase activity	59	0.001415
	GO:0003810	protein-glutamine gamma-glutamyltransferase activity	5	0.006306
	GO:0004714	transmembrane receptor protein tyrosine kinase activity	11	0.007039

On applying these DM CpG-associated genes, it was confirmed that the genes were expressed differently, and the brindle cattle (brindle color) identified the difference in the expression of many genes. In other words, 002072558324 objects, with the highest expression of brindle color, showed

hypomethylation of 3,332 genes and hypermethylation of 4,219 genes after growth, while 002072550199 objects, which had the lowest expression of brindle color, showed hypomethylation of 1,460 genes and hypermethylation of 865 genes after growth (Table 2).

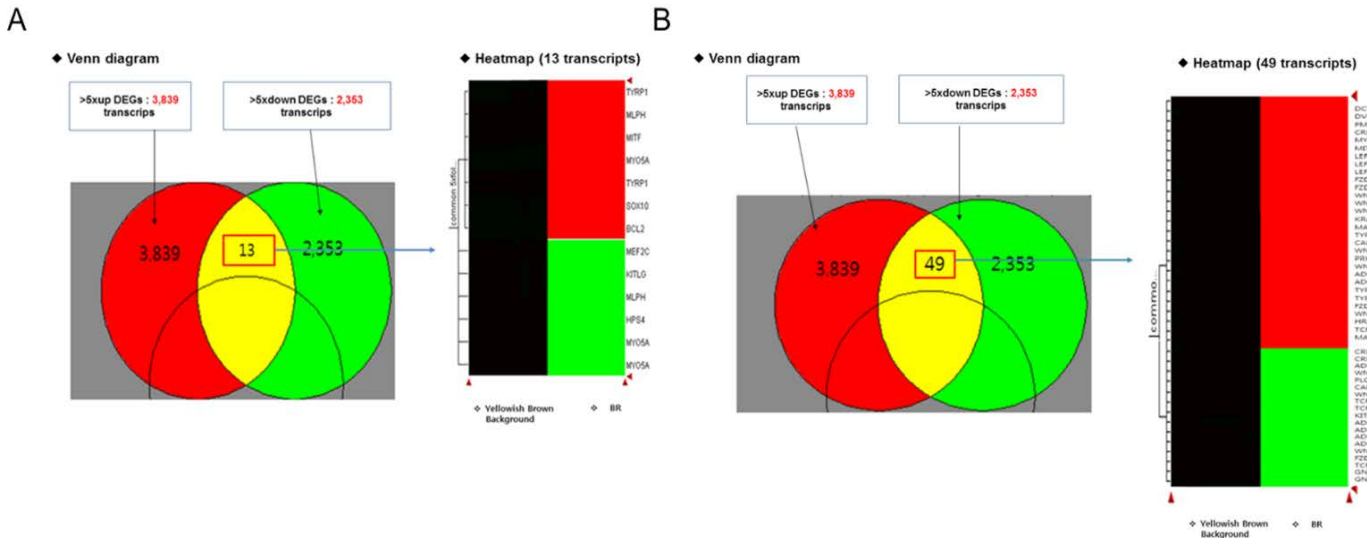


Figure 4: Profile analysis of the skin transcriptome in the brindle coat-Yellow zone: melanocyte-associated gene (common in >5×fold), A: melanocyte differentiation: 13 transcripts, B: melanogenesis differentiation: 49 transcripts.

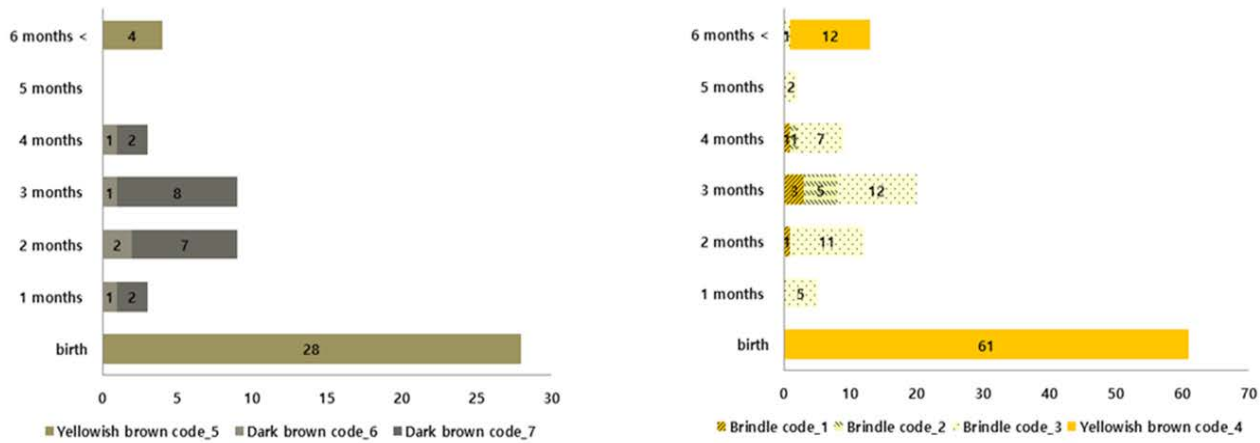


Figure 5: Changes in coat color depending on the growth stage.

Table 2: Differentially methylated CpGs with different coat color patterns.

Sample		# Differentially methylated CpGs (FDR<0.01, methylation difference>25%)		
Control	Case	All	Hyper	Hypo
002072550199_160321_1	002072550199_160901_2	2325	865	1460*
002072550211_160321_1	002072550211_160901_2	2359	1416*	943
002072550318_160420_1	002072550318_160901_2	1493	773	720
002072550359_160420_1	002072550359_160901_2	3133*	2576	557
002072558324_160317_1	002072558324_160902_2	7551**	4219**	3332**
brown	brindle	1395	893	502

*,** Different letters within the same column represent a significant difference at P < 0.05 and P < 0.01 respectively.

Table 3: KEGG pathways for DM CpG-associated genes- brown vs. brindle (FDR <0.001, methylation difference >25%).

Term	Count	%	PValue	Genes	Fold enrichment	FDR
bta04916: Mel-anogenesis	24	1.355167	7.04E-06	WNT5A, TYRP1, HRAS, GNAI2, MAP2K2, GNAI1, ADCY5, CREBBP, FZD1, KITLG, TCF7L2, WNT1, EDNRB, PLCB3, WNT3, PLCB4, CALM, WNT11, GNAS, CALML5, PLCB1, ASIP, CALM2, CALM1	2.828159	0.009283
bta04310: Wnt signaling pathway	25	1.411632	5.25E-04	WNT5A, NKD1, CTBP2, CREBBP, FZD1, SMAD4, TCF7L2, WNT1, PLCB3, CCND1, SOST, PLCB4, WNT3, PSEN1, SFRP1, PPP3CC, WNT11, MAPK8, SOX17, BAMBI, PLCB1, AXIN2, MYC, CUL1, NFATC1	2.12886	0.690278
bta04010: MAPK signaling pathway	27	2.045455	0.009372	PTPN7, FGFR1, HRAS, FGFR3, PDGFA, NF1, GNA12, MAP4K1, MAPK11, TAB1, TGFB2, PRKCB, RPS6KA5, RPS6KA6, DUSP2, DUSP1, MAPK3, MAPK8, PAK1, MAPK7, MAP3K14, GADD45B, MYC, MAP2K6, CACNA1B, NFATC1, FGF4	1.684266	11.67573
Thyroid hormone signaling pathway	15	1.136364	0.010108	FXD2, HRAS, PIK3CB, RXRA, CREBBP, RCAN1, PRKCB, HIF1A, DIO3, MAPK3, MED27, GATA4, RHEB, MYC, SLC9A1	2.128103	12.53706

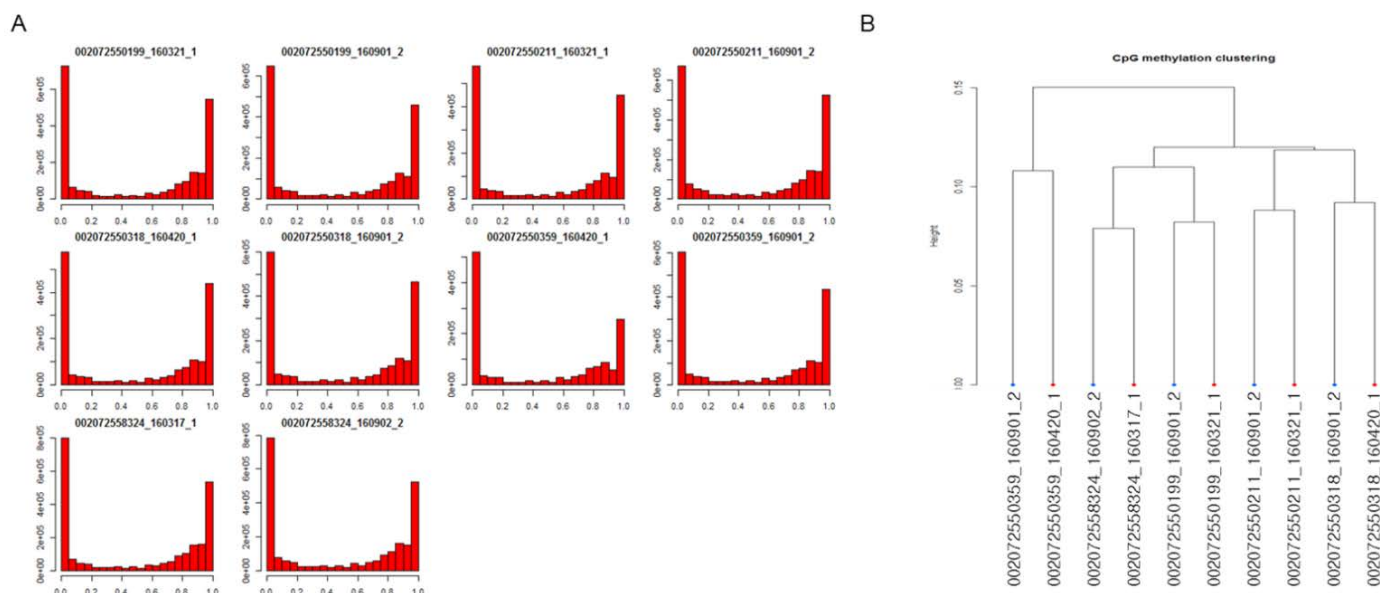


Figure 6: Methylation level distribution among brindle cattle- A: methylation level, B: clustering by CpG methylation.

THE CHANGE IN DNA METHYLATION IN INDIVIDUAL GROWTH STAGES

Hypomethylation in 201380 Section 1 of the CpG chromosome was done by 27.11% of 002007255019 objects, while hypomethylation in the 44256011 sections of the CpG chromosome 29 was done by 100% of 002072550211 objects at growth. Hypomethylation in 100178350 sections of the CpG chromosome 1 was done by 66.66% of 002072550318 objects, and hypermethylation in 48967783 sections of the CpG chromosome 11 was done by 62.5% of 002072550359 objects. Hypomethylation in 10195476 of the CpG chromosome 11 was done by 66.66% of 0020072550324 objects with distinctly expressed brindle color (Figures 6 and 7). Analysis of gene

variation among DNA methylated genes by the GO term in the growth process of brindle cattle showed that among the identified genes, the Wnt, MAPK genes associated with melanogenesis, and thyroid hormone related genes were the main cause. Hypomethylation of MAPK and thyroid hormone related genes appeared to occur at a very high rate (Table 3).

DISCUSSION

The difference in the phenotype (heterozygote/homozygote) of the MC1R gene in cattle has been reported to influence the binding of α-MSH (Chung et al., 2000; Seo et al., 2018). This variation also plays an important role in the

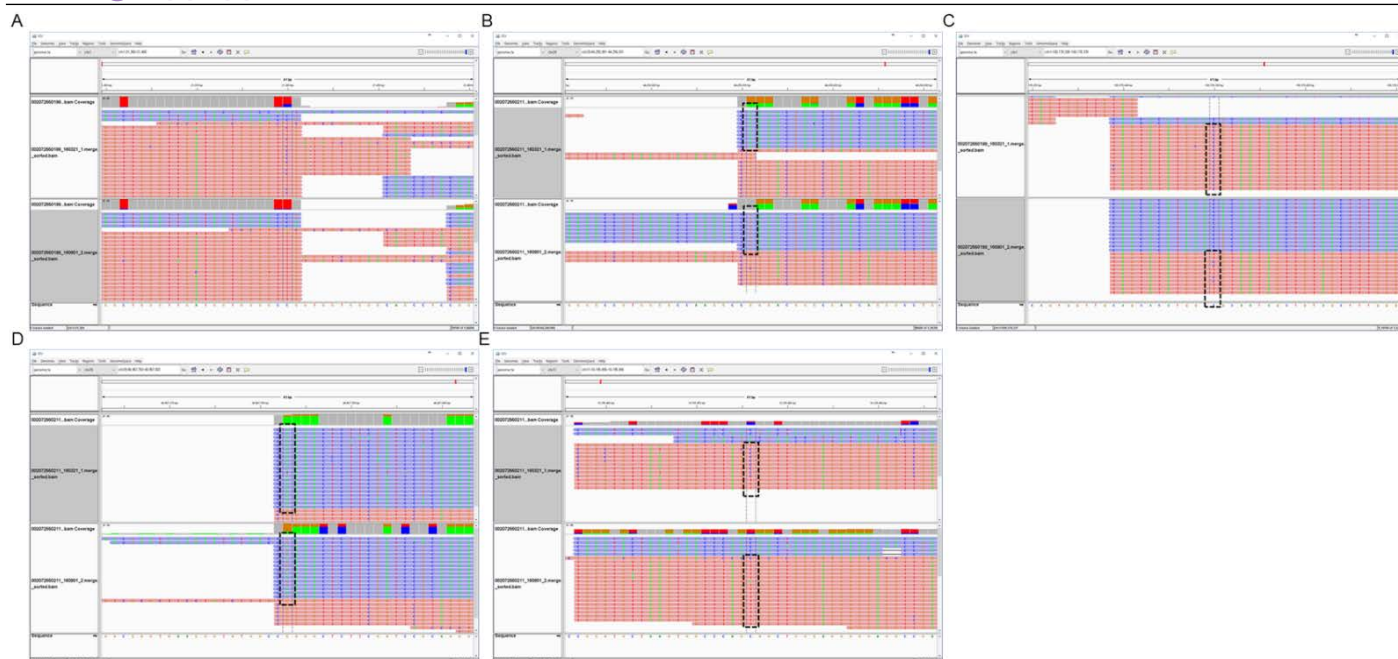


Figure 7: Hypomethylation in the brindle section- A: CpG chr1:21380(+) hypomethylation at brindle 002072550199, B: CpG chr29:44256011(-) hypermethylation at brindle 002072550211, C: CpG chr1:100178350(+) hypomethylation at brindle 002072550318, D: CpG chr11:48967783(-) hypermethylation at brindle 002072550359, E: CpG chr11:10195476(+) hypomethylation at brindle 00207255032.

gene expression of final TYR, TYRP1, and DCT during the process of melanogenesis. However, the variation in MC1R phenotype is either caused by the genetic action or the DNA methylation of the epigenome. Hence, this mechanism of melanogenesis was believed to play a role in the formation of the final metabolite of melanin (Kim et al., 2018). Therefore, this study analyzed whether changes in coat color of brindle cattle and the variation in related genes were characteristically or specially associated with the mechanism of melanogenesis, depending on the type of pigmentation within the melanocytes in brindle cattle (Ishii et al., 2017). Analysis of the expression of genes of the black and yellow color sections of the brindle cattle (brindle color) demonstrated that the expression of major factors such as TYR and DCT, among the genes related to the melanogenesis, in the yellow and brindle sections was more than five times higher than that in the black sections. In other words, this result showed that the action of the melanocytes in the black and yellow sections was different. In this study, as evidence of genetic variation related to melanogenesis, analysis of methylation variation in DNA from blood collected from brindle cattle at the age of less than one month, when the coat color had not formed, and six months, when the coat color had formed, was performed. It demonstrated a 2x75 bp read set of around 80% of the Q30 base of 40 to 70 million reads. Analysis of the gene sequence of CpG formed by Wnt, MARK, and thyroid hormone signaling, which are the major mechanisms of melanogenesis, confirmed that the epigenesis of TYR and MARK-linked genes was very high. In other words, coat color variation in brindle cattle, is believed to be activated

as the young cattle grows into the adult phase. The control mechanism is considered to accelerate the expression of coat color. The results of the study by Choi et al. (2013) identified base sequence variations in about 18,000 genes in the results of NGS of brindle and yellow color and suggested the possibility of specific markers for brindle cattle. Additionally, epigenomic variation was found to occur during the mechanisms of melanogenesis, making it possible to develop epigenetic markers. Therefore, the results of this study confirmed that coat color due to melanocyte and keratinocyte pigments in brindle cattle was determined by the different variations in coat color of different species, including dairy.

CONCLUSION

The classification of breeds according to the phenotype of cattle is a very important factor of securing genetic resources in many countries. But so far, the exact understanding of the phenotype of a cattle's coat color remains unknown. Therefore, we analyzed the changes in melanogenesis according to the epigenetics of cattle. Our study results proved, the Korean brindle cattle showed an increase in melanogenesis by both MC1R and MARK signaling and the cell cycle active mechanism, resulting in variations in coat color. Additionally, analysis of expression patterns of epigenetic genes of changes in coat color during the growth process of calves in brindle cattle showed that there were changes in the MARK-related and melanogenesis-associated major factors in brindle color formation. The results of this study could be used as important base data

for further study of Korean brindle cattle and further demonstrate the unique characteristics of this breed.

AUTHORS CONTRIBUTION

All authors contributed equally.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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