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Changes in methylation of genomic DNA from chicken immune organs in response to H5N1 influenza virus infection

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ABSTRACT. DNA methylation is an important epigenetic modification in eukaryotes, which plays a significant role in regulating gene expression. When the host is invaded by the influenza virus, gene expression is regulated via changes in DNA methylation levels or patterns, leading to the activation or suppression of relevant signaling pathways or networks, triggering a series of immune responses against viral invasion. Here, we investigated the changes in genomic DNA methylation in the immune organs of chicken infected with H5N1 influenza virus. Genome-wide DNA methylation levels in the spleen, thymus, and bursa of Fabricius of specific pathogen-free (SPF) chicken infected with the Guangdong (G-H5N1) and Anhui (A-H5N1) H5N1

strains, and water (control) were analyzed by fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP). The results indicated that total DNA methylation levels did not differ between spleen genomic DNA in chicken treated with different viral strains and the control ($P > 0.05$). However, the total DNA methylation levels were significantly upregulated in the thymus ($P < 0.01$) and bursa ($P < 0.05$) of chicken in the A-H5N1 group compared to those in the G-H5N1 and control groups. These results provide a basis for the screening of avian influenza-resistance genes or methylation markers, analyzing the epigenetic regulation mechanisms of avian influenza, and performing selective breeding for disease resistance.

Key words: Avian influenza; H5N1; Immune organs; DNA methylation; F-MSAP

INTRODUCTION

Regulation of gene expression in eukaryotes is achieved as a result of synergy between the genomic, transcriptional, post-transcriptional, translational, and post-translational levels (Meikar et al., 2013; Holoch and Moazed, 2015; Lomniczi et al., 2015; Schübeler, 2015). DNA methylation, which occurs after DNA replication and before transcription, is one of the methods with which the gene is accurately regulated. The DNA methylation process, wherein S-thio-adenosylmethionine serves as a methyl donor and the methyl group is transferred to the fifth carbon atom of cytosine, is catalyzed by DNA methyltransferase (Auclair and Weber, 2012). DNA methylation plays an important role in X chromosome inactivation (Mohandas et al., 1981; Cotton et al., 2015), genetic imprinting (Li et al., 1993; Denomme and Mann, 2013), transposon silencing (Law and Jacobsen, 2010), cell differentiation (Meissner et al., 2008; Bock et al., 2012; Smith and Meissner, 2013; Kulis et al., 2015), maintenance of chromatin structure (Razin, 1998), embryonic development (Anifandis et al., 2015; Aoshima et al., 2015), cancer genesis (Kulis et al., 2013; Paska and Hudler, 2015), among other processes.

DNA methylation affects gene expression by regulating transcription initiation. Essentially, DNA methylation involves the establishment and maintenance of methylation and removal of methyl groups, processes that are catalyzed by corresponding enzymes. A gene that must be "silenced" is immediately methylated resulting in reduced or lack of expression; however, the silenced gene can be re-activated by demethylation, which in turn restores its transcriptional activity. DNA methylation occurs at the cytosine residues of CpG islands, commonly located in the promoter or first exon region of the gene. Gene expression is negatively correlated with promoter hypermethylation, but is positively or weak-negatively correlated with methylation within the gene (Li et al., 2012; Huang et al., 2014; Hsieh, 2015). Methylation of the cytosine residues at the CpG islands leads to a change in the binding of transcription factors or the chromatin structure, which causes inhibition of gene transcription, thereby regulating the gene expression and participating in a series of life events (Weber et al., 2005).

Avian influenza virus (AIV) belongs to the influenza type A virus family, which infects vertebrates. There are several subtypes of highly pathogenic AIV (HPAIV), including subtypes H5 and H7. Ten H5N1 HPAIV clades have been isolated and identified (WHO/OIE/FAO H5N1 Evolution Working Group, 2008). When H5N1 infections are prevalent, the AIV genome may

undergo mutations and integrate with other viruses in the host cells, readily resulting in the formation of new antigenic epitopes. As a result, previously developed vaccines could fail to act, resulting in disease development and substantial economic loss to the poultry industry. Additionally, H5N1 AIV alters the antigenic determinants, thereby expanding the scope of potential hosts, posing serious threats to the health of humans and other animals.

At present, research into H5N1 AIV is mainly focused on the pathogenesis, virulence, and prevalence and development of vaccines against the disease (Alkhamis et al., 2015). Few studies have attempted to investigate the regulation of relevant gene expression in the host during AIV infection, through changes in DNA methylation, in order to resist the viral invasion. The results of this study will provide a new perspective towards understanding the epigenetic regulatory mechanism of avian influenza and exploring functional genes that determine resistance traits and their patterns of regulation. This study will also provide a basis for future study in breeding for disease resistance.

MATERIAL AND METHODS

Ethics statement

Animal experiments were performed in accordance with the guidelines on animal care established by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Animals and strains

BWEL-SPF chicken and subtypes of the H5N1 AIV strains, including the Guangdong strain (A/Goose/Guangdong/1/96, H5N1; G-H5N1) and Anhui strain (A/Duck/Anhui/1/2006, H5N1; A-H5N1), were provided by the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Inoculation

AIV inoculations were carried out in the P3 laboratory of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Fifteen 4-week-old BWEL-SPF chicken were randomly divided into three groups (N = 5 per group). The chicken received 10^6 EID_{50} (50% egg infectious dose) G-H5N1, A-H5N1, or ddH_2O per 0.1 mL, using nose drops. The spleen, thymus, and bursa of Fabricius were collected from the chicken in the P3 laboratory 10 days after inoculation.

Genomic DNA extraction and purification

Genomic DNA (gDNA) was extracted according to the method described by Yang et al. (2011). The extracted gDNA was purified using the MicroElute DNA Clean-Up Kit (Omega, Hartford, CT, USA) according to the manufacturer protocols.

Enzyme digestion

gDNA purified from the same type of tissue of different individuals in the same group

were pooled. Each DNA pool was simultaneously subjected to an enzymatic digestion reaction using *Eco*RI + *Msp*I and *Eco*RI + *Hpa*II. The reaction mixtures were comprised of 4.0 µL gDNA (500 ng/µL), 10 U *Eco*RI, 10 U *Hpa*II/*Msp*I, 4.0 µL 10X Tango™ buffer, and 12.0 µL ddH_2O. The samples were digested at 37°C for 12 h.

Enzyme ligation

The digested products were ligated with 2.0 µL 10X buffer, 5 pmol *Eco*RI adapter, 50 pmol *Hpa*II-*Msp*I adapter, and 1.0 U T4 ligase at 37°C for 12 h. The adapters and primers for *Eco*RI and *Hpa*II/*Msp*I were previously described by Yang et al. (2011), with some modifications (Table 1).

Table 1. Adapter and primer sequences used for F-MASP analysis.

Primers/adapters	Sequence (5'-3')
*Eco*RI adapter	5'-CTCGTAGACTCGTACC-3'
	3'-CATCTGAGCATGGTTAA-5'
*Hpa*II/*Msp*I adapter	5'-GACGATGAGTCTAGAA-3'
	3'-CTACTCAGATCTTGC-5'
E+1 primers (PreAmp)	5'-GACTGCGTACCAATTC+A-3'
HM+1 primers (PreAmp)	5'-GATGAGTCTAGAACGG+T-3'
E+2 primers	5'-GACTGCGTACCAATTC+AAC-3'
	5'-GACTGCGTACCAATTC+AAG-3'
	5'-GACTGCGTACCAATTC+ACG-3'
	5'-GACTGCGTACCAATTC+AGT-3'
	5'-GACTGCGTACCAATTC+ATC-3'
	5'-GACTGCGTACCAATTC+ACT-3'
	5'-GACTGCGTACCAATTC+AGA-3'
	5'-GACTGCGTACCAATTC+ATA-3'
HM+2 primers	5'-FAM[1]-GATGAGTCTAGAACGG+TAC-3'
	5'-FAM -GATGAGTCTAGAACGG+TAG-3'

[1]Primer was labeled with the blue fluorescent dye 5-FAM (5-carboxyfluorescein).

Pre-amplification

The ligation products diluted 16-fold functioned as the template for pre-amplification; E+1 and HM+1 were used as the primers for this reaction (Table 1). The reaction conditions were set as follows: 94°C for 5 min; followed by 30 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min.

Selective amplification

For selective amplification, pre-amplification products diluted 10-fold were used as the template; E+2 and FAM-labeled HM+2 primers (16 pairs of primers were obtained using different combinations of 2 HM+2 primers and 8 E+2 primers; Table 1) were used. The reaction conditions were set as follows: denaturation at 94°C for 5 min; 13 cycles of denaturation at 94°C for 30 s, annealing at 65°C (with a decrement of 0.7°C after each cycle) for 30 s, and extension at 72°C for 1 min; 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 10 min.

The products of selective amplification were electrophoresed on a 2.0% agarose gel. DNA methylation polymorphism was analyzed using an ABI377 sequencer (American Applied Biosystems, Foster City, CA, USA).

Statistical analysis

gDNA methylation levels within the immune organs of different groups of chicken were statistically analyzed using SPSS 16.0 (IBM, Armonk, NY, USA). The significance of the observed differences was examined using one-way analysis of variance (ANOVA), followed by the Duncan LSD test.

RESULTS

gDNA digestion

*Hpa*II and *Msp*I are a pair of isoschizomers with the same recognition site, CCGG. However, these two enzymes exhibit different levels of sensitivity to cytosine methylation in the genome (Table 2). *Hpa*II cannot cut the CCGG site when the second cytosine is methylated (full methylation), while *Msp*I cannot cut the CCGG site when the first cytosine is methylated (hemimethylation). Therefore, the fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) technique detects different digestion patterns in the genome to identify gDNA methylation, ultimately reflecting the methylation levels and patterns of CCGG sites in the genome.

Table 2. Methylation sensitivity and restriction patterns of the *Hpa*II and *Msp*I isoschizomers.

Methylation status	Enzyme sensitivity			
	*Hpa*II	*Msp*I	H	M
CCGGC**C**GG GGCCGGCC	Active	Active	+	+
CCGG GGCC	Active	Inactive	+	-
C**C**GG GG**C**C	Inactive	Active	-	+

H and M indicate the enzyme combinations of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively; -: band absent; +: band present. Underlined: methylated cytosine.

The digestion results of gDNA are shown in Figure 1. The smear was relatively uniform, without obvious single fragments, indicating that the digestion was sufficient.

Pre-amplification

The results of pre-amplification are presented in Figure 2. The smear was relatively uniform in the lanes, and the majority of DNA fragments were smaller than 1500 bp in size; however, a few pre-amplification products exceeded 2000 bp in length.

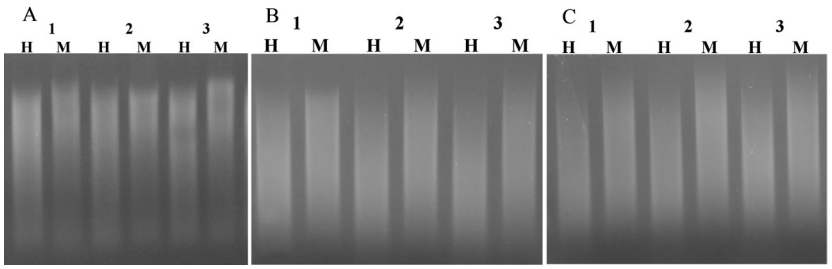

Figure 1. Agarose gel electrophoresis of digested gDNA from spleen, thymus, and bursa of Fabricius. **A. B.** and **C.** Electrophoretic images of the chicken spleen, thymus, and bursa of Fabricius, respectively; *lanes 1*, *2*, and *3* represent the A-H5N1, G-H5N1, and control groups. H and M refer to digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively.

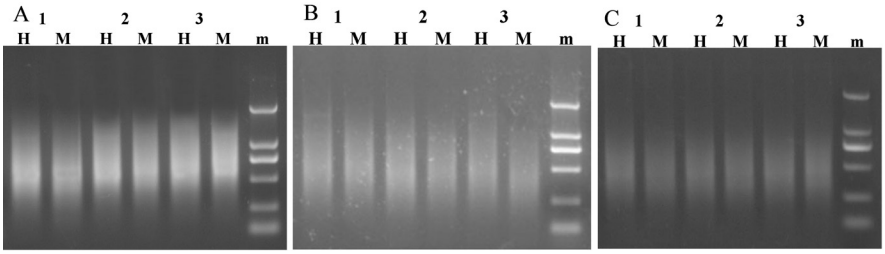

Figure 2. Agarose gel electrophoresis of pre-amplification products of the spleen, thymus, and bursa of Fabricius. **A. B.** and **C.** Electrophoretic images of the chicken spleen, thymus, and bursa of Fabricius, respectively; *lanes 1*, *2*, and *3* represent the A-H5N1, G-H5N1, and control groups. H and M refer to digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I. *Lane m*: DNA DL2000 marker.

Selective amplification

The results of selective amplification are illustrated in Figure 3. The fragments were diverse and clear, mostly ranging from 100 to 500 bp, indicating the efficient of amplification, without the loss of DNA fragments.

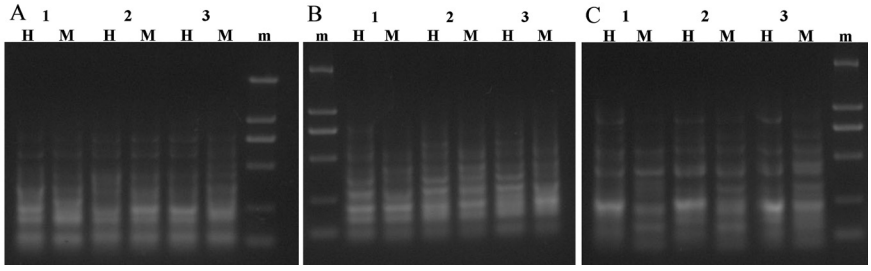

Figure 3. Agarose gel electrophoresis of selective amplification products of the spleen, thymus, and bursa of Fabricius. **A. B.** and **C.** Electrophoretic images of the chicken spleen, thymus, and bursa of Fabricius, respectively; *lanes 1*, *2*, and *3* represent the A-H5N1, G-H5N1, and control groups. H and M refer to digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I. *Lane m*: DNA DL2000 marker.

F-MSAP results

The products of selective amplification with the 16 pairs of fluorescently labeled primers were subjected to electrophoresis using an ABI377 sequencer to obtain fluorescence spectra illustrated in Figure 4.

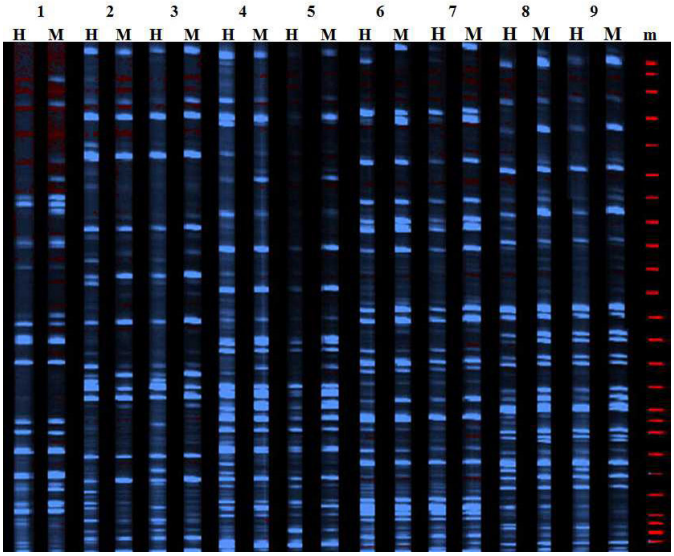

Figure 4. F-MSAP methylation profiles of gDNA from immune organs. *Lanes 1-3*, *4-6*, and *7-9* represent the F-MSAP profiles of the chicken spleen, thymus, and bursa of Fabricius, obtained using the primer combinations H-M+TAG and E+AAC. Lanes 1, 4, and 7 represent the A-H5N1 group; lanes 2, 5, and 8 represent the G-H5N1 group; and lanes 3, 6, and 9 represent the control group. H and M refer to digestion with *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I. *Lane m*: GeneScanTM-500ROXTM size standard.

Statistics for the F-MSAP data

The obtained fluorescence spectra indicate that the lanes were first corrected using GENESCAN™ 3.0, with an internal control. Subsequently, the size and location of DNA fragments were determined based on the fluorescence signals detected in the lanes using GeneScan™-500ROX™, and the data converted to an Excel spreadsheet using GENESCAN™ 3.0. Finally, the raw data were converted to values of 0 and 1, indicating the absence and presence of an amplified fragment at a specific position, respectively. The statistics for the F-MSAP data from chicken spleen, thymus, and bursa of Fabricius, obtained using 16 pairs of primers, are presented in Tables 3, 4, and 5.

Differences in gDNA methylation in the spleen, thymus, and bursa of Fabricius of chicken from different groups

The results of ANOVA of genome-wide DNA methylation levels in the various immune organs of the chicken are shown in Table 6. We observed no significant differences between the total DNA methylation levels in the chicken spleen of different groups ($P > 0.05$).

Table 3. F-MSAP statistical results of the spleen.

Group	Types	Primer number and the corresponding amplified fragment															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SPF	Full-methylated bands	23	19	20	23	19	11	10	16	27	21	22	18	18	17	12	7
	Hemimethylated bands	22	13	23	25	22	16	21	19	22	21	12	22	15	17	12	13
	Methylated bands	45	42	43	48	41	27	31	35	49	42	34	40	33	34	24	20
	Unmethylated bands	25	36	30	28	26	34	33	33	21	25	25	26	29	28	27	33
G-H5N1	Full-methylated bands	26	18	16	21	33	23	14	17	10	21	13	22	19	22	5	8
	Hemimethylated bands	19	19	18	14	13	15	12	17	16	17	24	24	11	26	17	11
	Methylated bands	45	37	34	35	46	38	26	34	26	38	37	46	30	48	22	19
	Unmethylated bands	27	37	36	32	19	33	34	26	30	31	24	26	31	31	33	26
A-H5N1	Full-methylated bands	25	12	12	21	19	19	5	21	22	16	10	18	25	25	11	11
	Hemimethylated bands	27	13	14	24	21	18	13	32	18	16	20	26	34	20	10	9
	Methylated bands	52	25	26	45	40	37	18	53	40	32	30	44	59	45	21	20
	Unmethylated bands	19	29	31	19	19	26	32	19	18	29	26	21	12	22	24	28

Table 4. F-MSAP statistical results of thymus.

Group	Types	Primer number and the corresponding amplified fragment															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SPF	Full-methylated bands	27	17	18	11	19	18	18	18	18	19	9	23	16	20	14	11
	Hemimethylated bands	24	9	13	19	18	16	16	22	14	18	14	14	24	28	12	8
	Methylated bands	51	26	31	30	37	34	34	40	32	37	23	37	40	48	26	19
	Unmethylated bands	24	37	32	32	34	34	33	29	28	24	24	25	26	24	25	31
G-H5N1	Full-methylated bands	35	10	21	16	10	12	22	13	16	16	10	35	14	15	13	11
	Hemimethylated bands	11	11	21	13	29	10	12	16	17	16	7	34	14	18	8	17
	Methylated bands	46	21	42	29	39	22	34	29	33	32	17	69	28	33	21	28
	Unmethylated bands	24	41	28	30	25	33	32	26	34	28	34	11	30	27	29	24
A-H5N1	Full-methylated bands	22	14	16	22	27	15	16	20	16	31	18	15	17	32	16	15
	Hemimethylated bands	22	14	11	16	22	17	20	19	16	41	18	17	17	30	16	16
	Methylated bands	44	28	27	38	49	32	36	39	32	72	36	32	34	62	32	31
	Unmethylated bands	16	28	28	19	14	21	23	21	23	9	22	18	22	9	20	24

Table 5. F-MSAP statistical results of bursa of Fabricius.

Group	Types	Primer number and the corresponding amplified fragment															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
SPF	Full-methylated bands	29	14	13	10	16	13	9	23	19	15	15	18	15	27	12	11
	Hemimethylated bands	23	15	14	17	17	20	14	24	18	16	12	17	13	24	6	11
	Methylated bands	52	29	27	27	33	33	23	47	37	31	27	35	28	51	18	22
	Unmethylated bands	22	33	36	34	30	32	36	29	27	31	25	22	26	21	29	35
G-H5N1	Full-methylated bands	21	18	15	20	20	11	14	13	13	6	7	20	10	23	14	8
	Hemimethylated bands	19	12	11	5	20	16	24	18	20	21	13	27	9	17	7	13
	Methylated bands	40	30	26	25	40	27	38	31	33	27	20	47	19	40	21	21
	Unmethylated bands	25	34	32	25	25	36	28	31	31	25	31	23	36	28	30	25
A-H5N1	Full-methylated bands	16	11	8	24	20	14	13	9	21	38	20	23	21	16	10	13
	Hemimethylated bands	21	13	12	20	14	11	16	25	24	38	22	22	12	14	11	9
	Methylated bands	37	24	20	44	34	25	29	34	45	76	42	45	33	30	21	22
	Unmethylated bands	21	25	32	17	22	24	25	19	20	7	21	17	20	27	24	29

Methylated bands = hemimethylated bands + full-methylated bands. 1-16 represent 16 primer pairs.

However, the total methylation of DNA extracted from the thymus and bursa of Fabricius of chicken in the A-H5N1 group were significantly higher than those seen in the G-H5N1 and control groups [$P < 0.01$ (thymus) and $P < 0.05$ (bursa)].

Table 6. Analysis of variance (ANOVA) results of genomic DNA methylation in immune organs.

Tissues	Methylation ratio (%)			F values	P values
	SPF	G-H5N1	A-H5N1		
Spleen	55.89 ± 2.16	53.49 ± 2.16	59.56 ± 3.44	1.325	0.276
Thymus	53.53 ± 2.07[a]	52.20 ± 3.21[a]	65.15 ± 2.87[b]	6.665	0.003
Bursa of Fabricius	51.80 ± 2.60[a]	50.33 ± 2.27[a]	59.80 ± 3.35[b]	3.412	0.042

Methylation ratio = methylated bands/total amplified bands; total amplified bands = unmethylated bands + hemimethylated bands + full-methylated bands. Superscript letters mean significant diferrences.

DISCUSSION

Methylation can be detected using three methods: genome-wide methylation detection, site-specific methylation detection, and identification of new methylation sites. A number of detection methods have been developed over the past decade (Shiraishi et al., 2004; Suzuki and Bird, 2008; Gupta et al., 2010; Mastan et al., 2012; Shan et al., 2012). F-MSAP is a technique used to detect DNA methylation at the whole-genome level. Methylation-sensitive restriction endonucleases do not cut methylated regions; therefore, F-MSAP amplifies the digested gDNA into fragments of different sizes, following which the methylation level and patterns are analyzed. Compared to traditional MSAP, F-MSAP uses fluorescently labeled selective primers, is efficient, sensitive, time-efficient, safe, and automatic (Yang et al., 2011). So far, F-MSAP has been used in biological, medical, and agricultural sciences, among others. In this study, F-MSAP was employed to detect genome-wide DNA methylation levels in the chicken spleen, thymus, and bursa of Fabricius. The methylated fragments detected using 16 pairs of selective primers were diverse, indicating that the F-MSAP detection method is efficient, sensitive, and reliable.

Epigenetics, which includes DNA methylation, histone modifications, and noncoding small RNA, is the study of changes in cellular phenotypes or gene expression, unrelated to changes in the DNA sequences (Sanders et al., 2015). DNA methylation regulates gene expression by two mechanisms: 1) 5-methylcytosine (5mC) methylation affects the interaction between protein factors and DNA. 5mC extends into the major groove of the DNA double-helix structure, which provides the binding site for a large number of protein factors and contains GC-rich sequences that can be recognized by transcription factors. However, 5mC methylation leads to the transcription factors being unable to recognize the GC sequences, thereby hindering the binding of specific transcription factors to the recognition site, further affecting the binding of transcription factors to the promoter region. 2) DNA methylation affects the structure of chromatin and alters its conformation. DNA methylation can cause changes in the chromatin structure in the corresponding region, causing the chromatin to become highly spiraled and condensed into clusters. As a result, the DNA endonuclease restriction sites and DNA enzyme-sensitive sites disappear, leading to loss of transcriptional activity and ultimately inhibiting gene expression.

In terms of structure and function, the vertebrate immune system can be divided into innate and adaptive immunity; adaptive defense includes humoral and cellular immunity (Jeurissen et al., 2000). Humoral immunity is responsible for generating specific antibodies, while cellular immunity is involved in cell-mediated cytotoxic reactions and delayed-type hypersensitivity reactions. The avian spleen, thymus, and bursa of Fabricius are extensively involved in both types of immune processes. Methylation of DNA does not alter its primary structure but regulates tissue-specific gene expression by affecting gene transcription and the

chromosomal configuration. In other words, gene expression is promoted by maintaining a state of non-methylation, demethylation, or hypomethylation, and conversely, gene expression is inhibited by methylation or by maintaining a hypermethylated state, thereby regulating gene function. A number of life processes (e.g., cell differentiation, embryonic development, X chromosome inactivation, and disease genesis) are regulated by the synergy between methylation and demethylation. Therefore, when the host is invaded by the H5N1 influenza virus, the body regulates associated gene expression through changes in DNA methylation levels or patterns, thereby activating or blocking a series of signaling pathways or networks related to humoral and cellular immunity, further triggering immune responses to resist the virus. Mukherjee et al. (2013) investigated the DNA methylation of promoter regions in 24 genes regulating inflammation or inflammation-related processes in human lung epithelial cells infected with 4 different AIV strains. Significant methylation differences were detected in the promoter regions of 7 genes; moreover, DNA methylation levels were altered to different degrees after infection with different strains (Mukherjee et al., 2013). Malodobra-Mazur et al. (2014) studied the regulatory activity of stearoyl-CoA desaturase in 22 genes involved in the inflammatory response in 3T3-L1 adipocytes, and reported that the overexpression or silencing of stearoyl-CoA desaturase 1 led to changes in the methylation of inflammatory genes, in turn causing changes in the related genes. In this study, the DNA methylation levels were altered in various immune organs following H5N1 infection, indicating that DNA methylation is involved in the regulation of gene expression in avian influenza. Additionally, the DNA methylation levels differed in particular immune organs infected with different H5N1 strains. Such differences might be related to the polymorphisms between different H5N1 subtypes, with amino acid drift presumably resulting in changes in virulence and pathogenicity.

Meanwhile, specific methylated fragments identified in the F-MSAP analysis of different groups were cloned, sequenced, and analyzed. We identified genes related to cell growth and differentiation (receptor tyrosine protein phosphatase), aging (aging-related nuclear prelamin A recognition factor), metabolic balance, hormone regulation and secretion (type I iodothyronine deiodinase), and transcriptional regulation (RNA-binding protein), in addition to genes associated with disease genesis. This finding indicates that interference from external factors results in the initiation or blocking of a series of reactions via changes in DNA methylation levels, thereby maintaining the body balance.

The trait or phenotype is a result of the interaction between genes and the environment (Church, 2011; Womack et al., 2012; McManus et al., 2014). Similarly, disease resistance is regulated by resistance genes (Kaiser, 2010), and individuals in the same environment exhibit varying degrees of resistance to diseases (either susceptible or resistant). There are three types of resistance genes: 1) single major genes, which control the expression of resistance traits; 2) polygenes (minor genes), which individually exert minor effects, with the trait being expressed upon joint action of multiple-minor genes; and 3) independent polygenes, which exist in smaller numbers and exert greater distinguishable individual effects. So far, few studies have analyzed the resistance genes in poultry. DNA methylation regulates the expression of resistance genes, thereby affecting the resistance of the individual to disease. Thus, a comparative analysis of differentially methylated genes between different H5N1 infection and control groups could help screen for resistance genes against avian influenza, or methylation markers for marker-assisted selection; this, in turn, could improve the efficiency and accuracy of stock selection, providing a reference for resistance breeding against avian influenza.

Conflicts of interest

The authors declare no conflict of interest.

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