Hypomethylation upregulates the expression of CD30 in lymphoma induced by Marek’s disease virus

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ABSTRACT Epigenetic modification is widely known to be involved in embryo development, aging, tumorigenesis, and many complex diseases. Both hypermethylation of CpG islands at the gene promoters and global hypomethylation are involved in the initiation and progression of carcinogenesis. However, only a small portion of hypomethylation occurs at gene promoters and leads to the overexpression of certain oncogenes. To determine whether DNA methylation plays a role in tumorigenesis of Marek’s disease, we selected one putative oncogene and 8 tumor suppressor genes from the gene expression profile for the analysis of DNA methylation variation. Four normal spleen tissues and 4 Marek’s disease virus-infected tumor spleen tissues were collected, and the methylation level of the promoter region of each gene was analyzed using MassARRAY. As a result, the promoter region of CD30 was hypomethylated and displayed a significantly higher expression in Marek’s disease virus-infected tumor spleen tissues compared with normal ones ($P < 0.05$). In neoplastic cells, CD30 was known to promote the survival and proliferation of T-cell lymphomas. This result suggests that activation of CD30 is possibly associated with the tumorigenesis of Marek’s disease.

Key words: hypomethylation, CD30, Marek’s disease lymphoma, tumorigenesis

INTRODUCTION

Marek’s disease is caused by an oncogenic poultry herpes virus with various clinical syndromes, depending on the virulence of the virus strain and the genetic resistance of the chicken. The study of Marek’s disease is of great importance because of its profound economical and scientific interest. It has been roughly estimated that the cost of Marek’s disease worldwide was in the range of $1 to 2 billion annually (Morrow and Fehler, 2004). And Marek’s disease has long been used as a model for the study of virus-induced lymphomagenesis, such as human herpes virus 1 and human herpes virus 3 (Osterrieder et al., 2006). In the past decades, several studies have focused on Marek’s disease virus (MDV) lymphomagenesis, and many MDV genes and gene products have been identified. Among them, a 339-amino acid-long Meq oncoprotein encoded within the repeat regions (MDV EcoRIQ) of MDV plays a critical role in oncogenicity, as its deletion abolishes the ability of the virus to induce tumors (Lupiani et al., 2004). In this study, we focused on methylation variation of genes in the chicken genome associated with Marek’s lymphomagenesis.

The study of DNA methylation in Marek’s disease has been carried out for a long time. However, our understanding of it is very limited due to the lack of efficient and reliable technology for epigenetics analysis and the shortage of genetic information about the chicken. Therefore, most early studies focused on the methylation status of MDV. Kanamori et al. (1987) found that the latent MDV1 DNA in MDV-transformed T-cell line was significantly methylated, using restriction endonuclease analyses. Other studies showed that the latent state of Marek’s disease might be influenced by methylation of the MDV genome. Fynan et al. (1993) found that the MDV genome within the avian leucosis virus-transformed cells was considerably methylated at 5′-CpG-3′ dinucleotides and demethylation by treatment with 5-azactidine results in enhanced MDV antigen expression and viral replication. Similar results were observed in the study of Hayashi et al. (1994). However, DNA methylation was not found to be involved in the downregulation of MDV lytic genes in MDV-induced tumors (Parcells et al., 1999). Not until recently, Yu et al. (2008) has compared the methylation level of DNA methyltransferase genes in Marek’s disease-resistant and susceptible lines with pyrosequencing. And they found that the methylation pattern for DNMT3a is tissue-specific, and it is age-specific for DNMT1.

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Human DNA methylation typically occurs in a 5′-CpG-3′ dinucleotide context. In chicken, 3 types of methylation (5′-CpG-3′, 5′-CpC-3′, 5′-GpC-3′) have been reported (Haigh et al., 1982; Xu, 2006). To explore the potential influence of DNA methylation on Marek’s disease, we evaluated the methylation variations of several putative oncogenes and tumor suppressor genes from the gene expression profile pertaining to Marek’s disease.

As a major component of the epigenome architecture, DNA methylation is associated with changes in gene expression without modification of the DNA sequence. Growing epigenetic studies and recent advances in technologies for evaluating DNA methylation (Xu et al., 2000; Ehrich et al., 2005; Schumacher et al., 2006; Tost and Gut, 2007; Wojdacz and Dobrovic, 2007; Wang et al., 2010), aided by functional genomics, have dramatically increased our understanding of the mechanisms of methylation regulation toward disease pathogenesis. Moreover, convincing evidence indicates that abnormal methylation can be used for the detection and diagnosis of diseases, prediction of responses to therapeutic interventions, and prognosis of outcomes (Levenson, 2010). The DNA methylation variation includes the loss of DNA methylation (hypomethylation) in repetitive elements, which is associated with chromosomal instability and gene activation (Aiba et al., 1989; Rohrs et al., 2009; Wolff et al., 2010), and the gain of DNA methylation (hypermethylation) of CpG islands in the promoter region, which corresponds to repression (Opavsky et al., 2007; Santos-Rebouças and Pimentel, 2007; Pogribny and Beland, 2009).

Many technologies have been developed for evaluating DNA methylation, but high-throughput applications and allele-specific methylation analysis techniques remain challenging. Sequenom MassARRAY platform is a novel approach based on a base-specific cleavage reaction, along with matrix-assisted laser desorption ionization time-of-flight mass spectrometric analysis (Coolen et al., 2007). Generally, MassARRAY is also a bisulfite-treatment-based method for DNA methylation analysis. After bisulfite treatment of genomic DNA, nonmethylated cytosine (C) will be converted into uracil (U), whereas methylated cytosine remains unchanged. The cytosine/thymine (C/T) variations in the amplification products will appear as guanine/ad- enine (G/A) variations in the cleavage products generated from the reverse stand by base-specific cleavage. The G/A variations lead to 16 Da per CpG site mass difference, which can be detected by the MassARRAY system. The relative amount of methylation is obtained by comparing the signal intensity between the mass signals of methylated and nonmethylated template in mass spectrum. This technology evaluated various parameters of epigenetic profiling, such as the influence of DNA sequence variations, amplification conditions, and sensitivity (Coolen et al., 2007; Ehrich et al., 2007, 2008), and it is expected to provide a more reliable result. Moreover, this high-throughput technique offers the longest possible read length (500–600 bp per reaction) and provides quantitative methylation analysis of up to 85% of the CpG sites in the amplicon of a single reaction. Ideally, it can use different algorithms to achieve the analysis of 5′-CpC-3′ methylation and 5′-GpC-3′ methylation, thus being a better choice for our present experimental setup.

**MATERIALS AND METHODS**

### Microarray

In this experiment, 150 one-day-old specific-pathogen-free White Leghorn chicks (obtained from the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science) were divided into 2 groups randomly. The tumor group of 100 chicks was infected intraabdominally with 2,000 plaque-forming units (PFU) of virulent serotype 1 MDV strain GA (provided by Dr. C. J. Liu from Harbin Veterinary Research Institute, Harbin, China). The normal group of 50 chicks was injected with the same dosage of diluent. The 2 groups were housed in separate cages in the same filtered-air, positive-pressure isolation room. All the survivors from the experimental group and the control group were dissected at the end of the trial period (60 d postinjection).

We used 4 normal spleens tissues (NoST) from noninfected chickens and 4 age-matched tumor spleen tissues (TuST) from MDV-infected ones (Lian et al., 2010) for both of the DNA methylation and RNA expression analyses in our present study. The total RNA was extracted using Trizol extraction method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The genome-wide expression of each element (probe) was assigned to 4 different comparisons as NoST (noninfected chickens) and TuST (MDV-infected chickens). The gene expression profile was then conducted using a chicken 44 K Agilent microarray (Bohao, Shanghai, China) to analyze gene expression changes with Marek’s disease on a genome-wide scale (L. Lian, L. Qu, N. Yang, China Agricultural University, Beijing, China, unpublished data). Subsequently, key differentially expressed genes were selected according to the P-value, fold change (FC), and biological functions. Finally, the tumor-related genes with relatively high FC were then subjected to methylation analysis, and genomic sequences of the candidate genes were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/).

### Candidate Genes for Methylation Analysis

In the microarray analysis, 5,397 differentially expressed genes were obtained from the gene expression profile. Genes for methylation analysis were chosen through several standards. First, genes that showed significant changes in gene profiling were selected. A total of 778 genes was significantly downregulated, and
627 genes were significantly upregulated between the control group (NoST) and the tumor group (TuST; \( P < 0.05; \text{FC} > 2 \)). Second, genes were selected by their function. The molecular function of the selected genes was predicted by gene ontology and pathway analysis based on the SBC analysis system (an online system utilizing R-software and various databases, such as gene ontology and KEGG and Biocarta databases for gene function prediction). Seventy-nine immune-related genes and especially tumor-related genes, such as genes correlated with cell apoptosis, proliferation, cell cycle regulation, and cell metabolism, were selected (50 decreased in gene expression profile and 29 increased). Finally, we looked for other methylation studies on these 79 genes with the hope of recognizing the genes that would most likely experience methylation changes. Nine most-possible candidate genes with relatively higher FC were finally chosen for further methylation analysis, which were the following: \( \text{TNFRSF8 (CD30)} \), \( \text{CD79B} \), \( \text{CCNA1} \), \( \text{TAC1} \), \( \text{LYZ} \), \( \text{GRPR} \), \( \text{GALNTL6} \), \( \text{COL14A1} \), and \( \text{GFRA2} \). The information of their gene expression profile is displayed in Table 1.

**DNA Methylation Analysis**

To investigate the methylation variations of the candidate genes in tumorigenesis, we collected 4 normal spleen tissues and 4 tumor spleen tissues with apparent lesions from the tumor phase of MDV infection. Genomic DNA was extracted by traditional phenol and chloroform methods. The quality and quantity of the genomic DNA samples were evaluated respectively by gel electrophoresis and NanoDrop ND-1000 (Wilmington, DE) spectrophotometer.

A total of 2 μg of genomic DNA from each sample was treated with sodium bisulfite using an EZ DNA methylation kit (Zymo Research, Orange, CA), and the modified DNA was amplified by PCR. The target regions were amplified using the primer pairs that were designed with Sequenom online EpiDesigner (http://epidesigner.com). Because there are 3 methylation types in chicken, the assay design was quite challenging due to the density of GpC and CpC in the candidate genes. We have focused on the transcription start site (here we assumed it begins where mRNA sequence starts) and attempted to cover 500 to 1,000 bp on either side with 3 to 4 amplicons. Some amplicons overlapped and covered similar regions to verify the reproducibility of the data. And some amplicons were located on the forward strand, whereas others were on the reverse strand. Each forward primer was tagged with a decamer (5’-AGGAGAGAG-3’), and each reverse primer had a T7-promoter tag (5’-CAGTATACGACTCAC TATAGGGAGAAGGCT-3’), and each reverse primer had a T7-promoter tag (5’-CAGTATACGACTCAC.TATAGGGAGAAGGCT-3’) for in vitro transcription. The amplicon primers for the gene that were finally identified with significant methylation changes are detailed in Table 2.

The PCR amplification on 25-μL samples was performed using the following conditions: hot start at 94°C for 15 min, followed by 45 cycles of denaturing at 94°C for 20s, annealing at 62°C for 30s, extension at 72°C for 1 min, and a final incubation at 72°C for 3 min. Unincorporated dNTPs were dephosphorylated by adding 2 μL of premix, including 0.3 U of shrimp alkaline phosphatase (SAP; Sequenom, San Diego, CA). The reaction mixture was incubated at 37°C for 20 min, and SAP was then heat-inactivated for 5 min at 85°C. After SAP treatment, 2 μL of the PCR products were used as a template for in vitro transcription, and RNase A cleavage (T cleavage) was used for the reverse reaction, following the manufacturer’s instructions. Subsequently, the samples were conditioned and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser RS1000 (Samsung, Irvine, CA), followed by spectral acquisition on a MassARRAY analyzer compact MALDITOF MS (Sequenom). Quantitative results for each CpG site or an aggregate of multiple CpG sites were generated by Epityper software version 1.0 (Sequenom).

**Quantitative Real-Time PCR**

The total RNA of 4 individuals from tumor spleen tissues and normal spleen tissues was extracted using the total RNA extraction kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer’s instructions. The first strand cDNA was synthesized from total RNA using M-MLV reverse transcriptase (Promega Biotech Co. Ltd., Beijing, China). Primers for \( \text{CD30} \) were verified and the PCR-amplified prod-

### Table 1. Key differentially expressed genes in Marek’s disease-infected tumor spleen tissues

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Symbol</th>
<th>P-value</th>
<th>T vs. N regulation</th>
<th>Fold change</th>
<th>Function/description</th>
</tr>
</thead>
<tbody>
<tr>
<td>777365/395090</td>
<td><strong>TNFRSF8</strong></td>
<td>0.0011</td>
<td>up</td>
<td>62.3692</td>
<td>Tumor necrosis factor receptor super-family, member 8</td>
</tr>
<tr>
<td>396276</td>
<td><strong>COL14A1</strong></td>
<td>0.0186</td>
<td>down</td>
<td>64.07</td>
<td>Collagen, type XIV, alpha 1 (undulin)</td>
</tr>
<tr>
<td>43673</td>
<td><strong>TAC1</strong></td>
<td>0.019</td>
<td>down</td>
<td>32.0285</td>
<td>Tachykinin, precursor 1</td>
</tr>
<tr>
<td>395993</td>
<td><strong>GFRA2</strong></td>
<td>0.0139</td>
<td>down</td>
<td>29.7432</td>
<td>Lysozyme (renal amyloidosis)</td>
</tr>
<tr>
<td>396218</td>
<td><strong>LYZ</strong></td>
<td>0.0311</td>
<td>down</td>
<td>26.5294</td>
<td>TNRALPHA</td>
</tr>
<tr>
<td>422560</td>
<td><strong>GALNTL6</strong></td>
<td>0.0015</td>
<td>down</td>
<td>21.1055</td>
<td>UDP-N-acetyl-alpha-d-galactosamine: polypeptide</td>
</tr>
<tr>
<td>419940</td>
<td><strong>CD79B</strong></td>
<td>0.0286</td>
<td>down</td>
<td>13.4469</td>
<td>N-Acetylgalactosamyl transferase-like 6</td>
</tr>
<tr>
<td>378928</td>
<td><strong>GRPR</strong></td>
<td>0.0088</td>
<td>down</td>
<td>9.5618</td>
<td>CD79B molecule, immunoglobulin-associated beta</td>
</tr>
<tr>
<td>418901</td>
<td><strong>CCNA1</strong></td>
<td>0.0027</td>
<td>down</td>
<td>6.8396</td>
<td>Cyclin A1</td>
</tr>
</tbody>
</table>

\(^1\text{T vs. N = tumor group versus normal group.}\)
Table 2. Primers used for sodium bisulfite sequencing and quantitative real-time (RT) PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>CpG¹</th>
<th>CpC</th>
<th>GpC</th>
<th>Size²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD30 amplicon-1</td>
<td>Epi-F</td>
<td>5′-TGTTGGGTGTTTTTGTTGAGTTAGTG-3′</td>
<td>14</td>
<td>35</td>
<td>47</td>
<td>593</td>
</tr>
<tr>
<td></td>
<td>Epi-R</td>
<td>3′-AATCAAATTGTCATTCTCATTGCT-5′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD30 amplicon-3</td>
<td>Epi-F</td>
<td>5′-AATTTAGGTGTTTTTGTTTGT-3′</td>
<td>6</td>
<td>21</td>
<td>27</td>
<td>409</td>
</tr>
<tr>
<td></td>
<td>Epi-R</td>
<td>3′-CCAACCTTTCCTTAAATCCCT-5′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD30</td>
<td>RT-PCR-F</td>
<td>5′-GGCCCTTCAGCACTGATGCTA-3′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RT-PCR-R</td>
<td>3′-ACACGCAAACACAAATAGTG-5′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>RT-PCR-F</td>
<td>5′-GAGAAATCTGCGTGACATCA-3′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RT-PCR-R</td>
<td>3′-TGCGCAATGAGAGGTTTCAAG-5′</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹The total number of CpG, CpC, GpC sites in each amplicon was included.
²Product size in bp.

RESULTS

Methylation Pattern of the Candidate Genes

Genes displaying altered expression levels in Marek’s disease could have undergone an increase or decrease of methylation, and those affected by methylation changes would be expected to have some methylated CpG sites in or in proximity of promoter regions. Here, we evaluated the methylation levels of the 9 candidate genes, which were all documented to display methylation variation in carcinogenesis. However, only the upregulated gene CD30 displayed significantly higher methylation with our normal group compared with the tumor group (Figure 1). The methylation ratio for each CpG site of CD30 promoter region was displayed in the Epigram generated by Epityper (Figure 2). Apart from CpG methylation, we also analyzed the CpC and GpC methylation in this experiment. However, these 2 methylation types were found to be individually specific. In general, no apparent pattern of change was observed between individuals.

Methylation Level of Each CpG Site in CD30 Promoter Region

A schematic diagram of CD30 promoter region was displayed to give an outline of each CpG site in the analyzed region. Three amplicons were designed around the initiation codon region. Only 2 amplicons covering 1,017 bp around the core promoter region were within good quality control parameters. Fifteen out of the 20 covered CpG sites were analyzed over the 2 amplicons (Figure 3). The transcription-factor binding sites were identified by using MATINSPECTOR (Quandt et al., 1995). And the core promoter motif and AP-1 binding sites were indicated respectively (Figure 3). In this schematic diagram, CpG site 3 (the third CpG site) and CpG site 4 were located in the middle of the promoter region. Meanwhile, CpG site 9 and CpG site 10 were in the Ap-1 binding sites.

We further compared the methylation differences of each CpG site within the CD30 promoter region between normal and tumor groups. As shown in Figure 4, methylation levels varied at the different CpG sites. The lowest level was observed on the CpG site 8 (0.06%), whereas the CpG site 3 had the highest methylation level (74.26%). The comparison between normal and tumor groups revealed that the methylation level of 11 out of 15 CpG sites were significantly higher in the normal group, including CpG site 10 at one of the Ap-1 binding sites (P < 0.05; Figure 4).

Gene Expression of CD30

To test whether methylation variations coordinate with expression change for CD30, we conducted quanti-
tative real-time PCR to evaluate the mRNA expression of CD30 in the same samples used for DNA methylation analysis. The result indicated that CD30 expression in the normal group was significantly lower than the tumor group \((P < 0.05; \text{Figure 5})\), which is congruent with the result in the gene expression profile.

**DISCUSSION**

Methylation of CpG islands located in the gene promoter region is a common mechanism of gene regulation. In particular, the study of hypermethylation in tumor suppressor genes has become an emerging area in exploring tumorigenesis. For example, promoter region hypermethylation of CD79B, COL14A1, CCNA1, IGFBP4, ST18, and TAC1 were reported to be associated with various carcinogenic processes (Jones and Laird, 1999; Jandrig et al., 2004; Sato et al., 2006; Ushmorov et al., 2006; Jin et al., 2007; Chang et al., 2009; Yang et al., 2009). Therefore, we investigated the methylation status of one putative oncogene and 8 tumor suppressor genes selected from the gene expression profile in Marek’s disease. As a result, the upregulated gene CD30 in the tumor spleen group showed a significantly lower methylation level. Meanwhile, we observed that the expression of CD30 in the tumor group is significantly higher than in the normal group. The results suggest the possibility that CD30 might experience epigenetic modification in initiating MDV lymphomas. For some of the selected genes that didn’t reach the significant level of methylation changes, it is possibly due to the limited sample number. Also, because we used the tumor tissues for the methylation analysis, the small portion of nontumor cells may decrease the chance for us to detect genes that underwent methylation modification. The genes that didn’t display any difference between different treatments could be regulated by other mechanisms instead of DNA methylation in Marek’s disease.

We identified 15 CpG sites out of an encompassed 21 CpG dinucleotides, located in the core promoter, exon 1 and intron 1 of CD30 that showed a significantly lower methylation level in MDV-infected tumor spleen tissues. Chicken CD30 was reported to have 15 predicted high-stringency AP-1 transcription factor binding sites (Burgess et al., 2004). In this study, CpG site 9 of the tumor group, which showed significant methylation difference compared with the normal group, was located in one of the AP-1 binding sites. Meq known as an MDV oncogene can form heterodimers with C-Jun, which was the strongest transactivator, by binding to AP-1 sites, leading to upregulation of interleukin-2 and CD30 (Levy et al., 2003; Levy et al., 2005). Burgess et al. (2004) also proved that Meq leads to CD30 overexpression.

**Figure 1.** The DNA methylation variation of the candidate genes. Methylation levels of candidate genes were compared between samples from a normal group and a tumor group. Kruskal-Wallis test was performed: **\(P < 0.01\). Methylation (\%) = the number of methylated CpG sites/(the number of methylated CpG sites + the number of unmethylated CpG sites).**

**Figure 2.** Profiling of site-specific CpG methylation was performed in the analyzed region of CD30 by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (produced by Epityper; Sequenom, San Diego, CA). Each line represents a CpG methylation profile of CD30 analyzed region from normal samples (N1–N4) and tumor samples (T1–T4). Colors of each circle represent the methylation level of each corresponding CpG unit. An \(x\) represents missing data at a given CpG site. The color bar (0 to 100%) indicates the percentage of methylation.
Figure 3. Map of CpG methylation sites in the core promoter, exon 1, and intron 1 of CD30. Individual CpG sites are indicated as vertical lines and numbered from 1 to 21. Two amplicons indicated as amplicon 1 and amplicon 2 were used to cover the analyzed CpG sites. Amplicon 1 ranges from 1 to 593 at the reverse strand, and amplicon 2 ranges from 608 to 1,017 at the forward strand. The bold CpG dinucleotides in this figure indicate the analyzed CpG sites. The circled CpG sites are the ones either out of the detection range of MassARRAY [some fragments are too small to be analyzed (<1,700 Da) or too large (>7,500 Da) after the cleavage] or the individual data point did not meet the quality criteria. The core promoter at the forward strand, Ap-1 binding sites at both strands, and low-complexity of repeat masker region (a region gained from repeat masker, which is a program that screens DNA sequences for interspersed repeats and low-complexity DNA sequences from RepBase library of repeats) at the forward strand are underlined as A, B, and C, respectively. Also, the initiation codon (ATG) at 853 is double underlined.
pression in Marek’s disease. Furthermore, Jones and Laird (1999) revealed that DNA methylation has been shown to reduce the binding affinity of sequence-specific transcription factors. These findings inferentially implicated that hypomethylation of CD30 could result in high affinity to meq/c-Jun dimer and consequently contributed to the high expression of CD30 in Marek’s disease. From the view of comparative biology, CD30 was reported as a highly conservative oncogene among chickens, humans, and mice (Burgess et al., 2004). And it was found that hypomethylated CD30 CpG islands of lymphocytes were associated with the pathogenesis of Hodgkin lymphoma and anaplastic large cell lymphomas (Watanabe et al., 2008). To sum up, there is a great possibility that the overexpression of chicken CD30 attributes to demethylation modification. How-

Figure 4. Methylation variations of each analyzed CpG site in CD30 core promoter, exon1, and intron 1 regions. Kruskal-Wallis test was performed: *P < 0.05 (n = 4 for each group).

Figure 5. Messenger RNA expression differences of CD30 in spleen between normal and tumor groups (two replicates for each reaction, n = 4 for each group).
ever, further research is warranted to determine the exact role of CD30 hypomethylation in tumorigenesis of Marek’s disease.

Among the non-CpG sites we analyzed, only 7 out of 639 CpC sites (1.10%) and 22 out of 832 GpC sites (2.64%) were methylated. For CpC and GpC types of methylation, mC residues were clustered and had higher methylation frequencies at certain regions, which might suggest that some positions are more susceptible to methylation than others. Similar findings were observed in transgenic Petunia hybrida (Meyer et al., 1994). In general, there was no methylation frequency difference of non-CpG sites between normal and tumor groups, which indicated that the non-CpG methylation in these genes may not be functionally correlated with tumor formation. Moreover, the methylated CpC and GpC sites were individually specific. This was prone to result from individual background differences, such as environmental causes. In mammals, non-CpG methylation was reported to be a result of Dnmt3 activity during gametogenesis, embryogenesis, and somatic tissue development as well as maintaining the stability of the genome (Ramsahoye et al., 2000; Yu et al., 2008). Because limited studies have been reported on methylation in chicken, the exact role of CpC and GpC methylation in Marek’s tumorigenesis remains to be elucidated.

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