INVESTIGATION

Genome-Wide Copy Number Variant Analysis in Inbred Chickens Lines With Different Susceptibility to Marek’s Disease

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ABSTRACT Breeding of genetically resistant chickens to Marek’s disease (MD) is a vital strategy to poultry health. To find the markers underlying the genetic resistance to MD, copy number variation (CNV) was examined in inbred MD-resistant and -susceptible chicken lines. A total of 45 CNVs were found in four lines of chickens, and 28 were potentially involved in immune response and cell proliferation, etc. Importantly, two CNVs related with MD resistance were transmitted to descendant recombinant congenic lines that differ in susceptibility to MD. Our findings may lead to better strategies for genetic improvement of disease resistance in poultry.

KEYWORDS CNV disease resistance Marek’s disease chicken

Marek’s disease (MD) is a worldwide problem for poultry industry, with annual loss reports of more than $1 billion (Davison and Nair 2004). MD is caused by Marek’s disease virus (MDV), which is an alphaherpesvirus belonging to the Mardivirus genus. There are three serotypes of MDV: serotype 1 (MDV-1), serotype 2 (MDV-2), and serotype 3 [HVT (Davison 2002; Lee et al. 2000; Tulman et al. 2000)]; however, only MDV-1 is pathogenic and causes a serious lymphoproliferative disease in susceptible chickens. MDV infection in host cells goes through a complex life cycle of multiple phases, defined as early cytolytic, latent, late cytolytic, and transformation phases (Calnek 1986, 2001). Currently, the control of MD mainly relays on vaccination. However, the vaccination efficacy has been experiencing erosion due to the development of the disease itself and emerging new strains of MDV with escalated virulence (Osterrieder et al. 2006). Therefore, improving genetic resistance to MD in chickens is a vital approach to augment current control measures.

Two highly inbred lines of chickens (lines 63 and 72, or L63 and L72) were reported to have different susceptibility to MD (Bacon et al. 2000), which were used to develop a series of recombinant congenic strains (RCSs) with varied susceptibility to MD (Bacon et al. 2000; Silva et al. 1996) and different responses to vaccination (Chang et al. 2010, 2011). The generation of the RCSs includes one cross between the L63 and L72, two backcrosses of the descendents to L63, followed by full-sib mating. Theoretically, each of the 19 RCSs on average contains approximately 87.5% of the L63 and 12.5% of the L72 genome. Until now, microsatellite markers were used to fingerprint the RCSs (Bacon et al. 2000). However, genetic and genomic variations potentially underlying the varied susceptibility to MD in these lines of chickens remain poorly understood.

Single-nucleotide polymorphisms, insertion/deletion polymorphisms, and copy number variations (CNVs) are the major sources of genetic and genomic structural variations in plants, animals, and human (Freeman et al. 2006). CNVs are defined as large DNA fragments with...
sizes ranging from 1 kb to several megabases deleted, inserted, duplicated, or translocated in genome (Beckmann et al. 2007). De novo and transmitted CNVs are found being involved in a number of diseases, including Crohn’s disease [with a lower copy number of the DEFB4 gene in humans (Fellermann et al. 2006)] and autistic spectrum disorder (Levy et al. 2011; Sebat et al. 2007). Notably, CNVs also are found to be related with gastrointestinal nematodes resistance and susceptibility in bovine (Hou et al. 2011).

In this study, we hypothesized that some CNVs in chicken contribute to MD resistance whereas others to MD susceptibility. Using two highly inbred lines of White Leghorn and two RCSs of the two inbred lines, which vary in resistance/susceptibility to MD, we performed an array comparative genomic hybridization CGH (aCGH) analysis of the four lines of chickens to test our hypothesis. To this end, we identified 45 CNVs in total by comparison among the four lines of chickens. The functions of genes located in CNVs were evaluated for their potential role MD resistance/susceptibility. Finally, we also compared our CNVs with the MD-related quantitative trait loci (QTL) regions.

# MATERIAL AND METHODS

## Animals

A total of six chickens were taken from L6₃, L7₂, RCS-L, and RCS-M, which are two recombinant congenic strains from L6₃ and L7₂ as mentioned above. The numbers of chickens sampled for this study from the lines were 2, 2, 1, and 1, respectively. The L6₃ and RCS-L are known resistant to MD, and the L7₂ and RCS-M are susceptible to MD (Bacon et al. 2000). The susceptibility of RCS-M is about half-way between the progenitor L6₃ and L7₂. RCS-L is one of most MD-resistant lines out of the RCS series, comparable with the background line L6₃ (H. M. Zhang et al., 2010, unpublished data). All of the chickens were kept in an Avian Disease and Oncology Laboratory (ADOL)-specific, pathogen-free facility until the bleeding at 15 month of age. All animals were handled closely following the United States Department of Agriculture, Agricultural Research Service, ADOL’s Guidelines for Animal Care and Use (revised April 2005) and the Guide for the Care and Use of Laboratory Animals published by Institute for Laboratory Animal Research (ILAR Guide) in 1996 (http://www.nap.edu/openbook.php?record_id=5140).

## Sample labeling and aCGH analysis

All test genomic DNAs were labeled with Cy3, which were cohybridized with the reference sample that is labeled with Cy5. The whole-genome tiling arrays galGal_WG_CGH, which contain 385k 50-75mer probes, were used to perform the aCGH analysis. The mean and median probe physical distance (bp) by using the mapping data from previous research (Heifetz et al. 2007, 2009; Liu et al. 2001; Vallejo et al. 1998). When there is more than 100 bp overlap between CNVs and QTL, we consider that region as shared region. However, as the QTL

### DNA extraction and validation of CNVs by quantitative real-time polymerase chain reaction (PCR)

Genomic DNA from 20 μL of red blood cells was extracted using the DNeasy Blood & Tissue Mini Kit (Qiagen). Primers for CNVs validation by quantitative real-time PCR were designed based on the probe information using Primer3.0 online primers designer system (http://frodo.wi.mit.edu/) and are shown in Supporting Information, Table S1. 

| Primer | Forward  | Reverse  |
|--------|----------|----------|
| 1      | 5'-TCCTGTACCTGAGGATGTGAA-3' | 5'-AGGAGCTTTGCTGCTG-3' |
| 2      | 5'-AGGAGCTTTGCTGCTG-3'       | 5'-ACCATGGGAGAAGGAAAT-3' |

RNA extraction and quantitative real-time RT-PCR

RNA was extracted from 20–30 mg spleen by RNeasy Mini Kit (Qiagen) and reverse transcribed by QuantiTect Rev. Transcription Kit (Qiagen). The primers for ENSGALG00000015816 quantitative real-time PCR are as follows: forward: TTGGACGGGGACCTTACAGAC; reverse: TCAGCCTGAGGAGTGTAAA. The iCycler iQ PCR system (Bio-Rad) and QuantiTect SYBR Green PCR Kit (Qiagen) were used to do the quantitative PCR to check the expression of ENSGALG00000015816. The with following procedures was run on the PCR system: denatured at 95° for 15 min, followed by 40 cycles at 95° for 30 sec, 55–60° for 30 sec, 72° for 30 sec, then extended at 72° for 10 min. The housekeeping gene GAPDH (forward: GAGGGTAGTGAAGGCTGCTG; reverse: ACCAGAAAACACCGTTGACG) was used to normalize the loading amount of cDNA.

## Gene content of the CNVs and Gene Ontology (GO) term analysis

The gene content of the CNVs was obtained by using the UCSC database (Karolchik et al. 2004). The Ensembl genes that overlapped with CNVs were extracted. The GO term accession and GO term name information for each Ensembl gene were obtained from BioMart in Ensembl (http://www.ensembl.org/biomart/martview). The enriched GO term was tested by using the hypergeometric distribution. Given a set of genes (N), m of them are attributed with a particular GO term, then the probability of k or more genes from the target gene set (n) with this GO term is as follows:

\[
P(X \geq k) = \sum_{X=k}^{\min(m,n)} \binom{m}{k} \binom{N-m}{n-k} \]

### CNVs and QTL overlapping

The MD-related QTL information was downloaded from Chicken QTLdb (http://www.animalgenome.org/cgi-bin/QTLdb/GG/index), which converted the QTL positions from genetic distance (cM) to the physical distance (bp) by using the mapping data from previous research (Heifetz et al. 2007, 2009; Liu et al. 2001; Vallejo et al. 1998). When there is more than 100 bp overlap between CNVs and QTL, we consider that region as shared region. However, as the QTL
regions are very big, all the CNVs that we found overlapped with QTL are contained in the QTL region.

Data releasing
The raw aCGH data have been deposited with the GenBank Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) database under the accession no. GSE38689.

RESULTS
Identification of CNVs in chickens with different susceptibility to MD
By using the accepted criteria of 0.5_5 to identify CNVs, we identified a total of 72 CNVs (Table S2) in four chicken lines. We found more CNVs shared between two individuals from the same line than those from two different lines. The percentage of shared CNVs between two individuals was 34.7% and 50% in L63 and L72, respectively; however, there were only 21.1% shared CNVs between L63 and L72. To avoid redundant CNVs, the 72 CNVs were merged into 45 CNVs, which span 3,297,038 bp in length of the chicken genome in four lines of chickens (Table 1 and Table S3). Among the CNVs, 33 (1,921,022 bp) were located on specific chromosome regions and 12 (1,376,016 bp) on uncharacterized chromosome regions (ChrAll_random). However, as with chrUnAll-containing sequences that lack mapping information to the genome, we should handle CNVs on chrUnAll with caution. We found a greater frequency of losses than gains (losses/gains: 31/14). The lengths of the CNVs were ranging from 9950 bp to 387,500 bp with a mean and median of 73,268 bp and 5000 bp, respectively. Because L63 and L72 are highly inbred lines and are also the progenitor lines of RCS-L and RCS-M, we found four shared CNVs with a total length of 769,202 bp among the lines (Table 1). Interestingly, these CNV regions overlap with some genes related with immune system. For example, several genes located in the CNVR deleted on Chr16, including ENSGALT00000004115 and ENSGALT0000028239, are major histocompatibility complex (MHC) class I antigen family genes.

Validation of CNVs
Before validating the CNVs, we compared our result with the published CNVs identified in White Leghorn chickens (Griffin et al. 2008; Wang et al. 2010). We found seven CNVs that overlapped with the reported CNVs (Table S4). The length of the overlap between these two sets of CNVs ranged from 17,814 bp to 207,851 bp. Then, a quantitative (Q)-PCR method was performed on selected CNVs, including one region (Chr2:49165364-49227941) found in all four chicken lines, which is considered as a transmitted CNV from L63 and L72 to RCS-L and RCS-M. Compared with RIF, most of the L63, L72, RCS-M, and RCS-L chickens tested had more than two copies in this region although several individuals showed different results, especially in RCS-M (Figure 1A). Then, 10 other CNVs were randomly chosen to for Q-PCR analysis. The regression analysis showed a strong linear correlation between the copy number identified by both Q-PCR and aCGH (Figure 1B).

Transmitted CNVs
Theoretically, for RCS-L and RCS-M, each on average should have a random sample of approximately 87.5% of the background L63 genome and 12.5% of the donor L72 genome, respectively. We found that approximately 91.7% and 66.7% of the CNVs in RCS-L and RCS-M were inherited from the progenitor lines L63 and L72, respectively (Table 2). The absolute numbers of the inherited CNVs in the two descendent lines were similar. There were seven CNVs that were shared between the L63 and L72, most of which were transmitted to

| Table 1 Distribution of CNVs in different chicken lines |
| Sample | CNV Count | Gain | Loss | Total Length, bp | Shared (Gain/Loss) | Shared Length, bp | Unique (Gain/Loss) | Unique Length, bp |
|--------|-----------|------|------|----------------|-------------------|-----------------|-----------------|-----------------|
| L63    | 22 (11)   | 8 (4) | 14 (7) | 1,915,598 | 6 (1/5) | 300,059 |
| L72    | 20 (10)   | 5 (2.5) | 15 (7.5) | 1,790,92 | 4 (2/2) | 869,721 |
| RCS-L  | 12 (12)   | 4 (4) | 8 (8) | 1,390,273 | 1 (0/1) | 15,000 |
| RCS-M  | 21 (21)   | 8 (8) | 13 (13) | 2,032,661 | 9 (3/6) | 413,217 |

The numbers in parentheses from columns 2 to 4 are CNV events per individual. The numbers in parentheses in column 6 and 8 are the gain/loss ratio of CNVs. Shared: CNVs shown in all four chicken lines; Unique: CNVs shown only in one chicken line. CNV, copy number variation.
the descendent RCS-L (6 of 7) and RCS-M (5 of 7) chickens. Approximately one-half of the CNVs that were unique for L63 were transmitted to RCS-L (7 of 15) and RCS-M (8 of 15). However, the CNVs that were unique for L72 were rarely transmitted: none to RCS-L and only one to RCS-M. In terms of the length of transmitted CNVs, approximately 92.7% and 88.6% of the CNV sequences in RCS-L and RCS-M were from L63, respectively, which is close to the theoretical expectation of 87.5%. Except for the transmitted CNVs there are also one CNVs in RCS-L and seven in RCS-M that are not shown in the progenitor chicken lines, which could be the potential lineage-specific CNVs.

Comparison of the CNVs between the resistant and susceptible chicken lines

Specifically, all genomic variations between the L63 and L72 could be considered as candidate regions for MD-resistance or -susceptibility. By comparison, we found that a total of 28 CNVs spanning 2,031,252 bp differed between the two lines of chickens (Table S3). These 28 CNVs encompass several functional genes, including AP2-associated kinase 1 (ENSGALT00000000026), amyloid beta (A4) precursor protein-binding, family A, and member 2 (ENSGALT00000006262). GO term analysis was then used to find the functions of the genes located in the differentially presented CNVs (Figure 2). For L63 unique CNVs, the deleted sequences contained genes that are related to immune response, MHC class I protein complex, antigen presentation, and presentation; the duplicated genes have functions of cell proliferation in midbrain, establishment of planar polarity, and Wnt-activated receptor activity. For the L72 unique CNVs, the lost genes are enriched in G-protein coupled receptor signaling pathway, flavin-containing monooxygenase activity, and NADP binding; the genes duplicated have functions of protein-glutamine gamma-glutamyltransferase activity, peptide cross-linking, and multicellular organism growth. As shown previously, we found many transmitted CNVs in RCS-L and RCS-M that have different phenotypes after MDV infection. The resistant CNVs and susceptible CNVs candidate that are transmitted to RCS-L and RCS-M respectively can be considered high confident CNVs for MD-resistance and MD-susceptibility. Thereafter, we found one MD-resistant candidate CNV that is a loss of genomic region on chromosome 19 spanning 50 kb and one MD-susceptible candidate CNV that is also a loss of genomic region on uncharacterized chromosomes region spanning 83.5 kb. One functional gene was found located on the MD-resistant candidate CNV, which is the general transcription factor III (GTF2I). However, only several ESTs were found in the MD-susceptible CNV, but no known functional genes were identified.

CNVs and gene expression

CNVs overlapped with genes were found regulating the gene expression (Stranger et al. 2007). To check the relationship between CNVs and gene expression in our CNVs, we extracted expression information from our previous microarray data (Yu et al. 2011) for genes located in the CNVs. In the microarray trial, we used the exact same chicken lines, L63 and L72, to perform gene expression analysis in chicken spleens in a MDV challenge experiment to check whether the host responses to the virus infection are different in chickens with different susceptibility to MD. The log-fold change of the differential gene expression between L63 and L72 was used for the following correlation analysis between gene expression and CNVs. In addition, the log-ratio of the signal density between L63 and L72 was also calculated from the raw data to show the different copy numbers between L63 and L72. For the CNVs that were shared in both L63 and L72 chickens, the expression of the genes in them are similar between these two chicken lines (Figure 3A). The expression of encompassed genes didn’t differ significantly between the two lines, and the correlation coefficient (rho = 0.09) was negligible (Figure S1). However, for unique CNVs, the gene expression differed significantly between L63 and L72. For most of genes, gain/loss of the copy number is related with the up-/down-regulation of the expression level (Figure 3A). The Pearson correlation analysis showed a high positive correlation (rho = 0.65) between the copy number and the gene expression for line-specific CNVs (Figure 3B). To further confirm the positive correlation of CNV and gene expression in our population, we picked up a candidate gene (ENSGALG00000015816) from the upper list and analyzed the copy number and gene expression level in our population.
As shown in Figure 4, when the copy numbers of the candidate gene are significantly greater in L72 chickens, the expression is significantly greater.

CNVs and MD-related QTL

It is reported that the QTL for MD resistance and susceptibility were mapped to several chromosomes, including GGA 1-5, 7-9, 15, 18, 26, Z, E21, and E16, by using the backcross or intercross populations (Heifetz et al. 2007, 2009; Liu et al. 2001; Vallejo et al. 1998). To identify whether common genomic variants were detected by using different methods, we compared our CNVs with the QTL regions. We found that those QTL regions overlapped with one of the shared CNVs, five CNVs only found in the resistant line, two CNVs only found in the susceptible line, and three of line-specific CNVs (Table 3). These overlapped CNVs were located on GGA 1, 2, 5, 9, 15, and Z, spanning 542,614 bp of the chicken genome. Among these overlapped regions, several functional genes were found, including LIM and senescent cell antigen-like domains 1 (LIMS1), myosin light chain kinase family, member 4 (MYLK4), frizzled family receptor 6 (FZD6), and brain and acute leukemia, cytoplasmic (BAALC).

DISCUSSION

In this study, we studied the CNVs in inbred chicken lines with different susceptibility to MD by aCGH. We found that four shared CNVs among the four chicken lines contain some immune-related genes, most of which are MHC class I antigen family genes. Because we used RJF as our reference to get these CNVs, this result indicates that compared with the wild chicken (RJF), the domesticated chicken lines (White Leghorn) have less copies of the immune related genes, which suggests that domestication may influence the immunity system of the chicken. In the meantime, when compared with reported CNVs identified in White Leghorns (Griffin et al. 2008; Wang et al. 2010), only seven shared CNVs were found, and most of the CNVs were different in our findings, which indicated different sample, breed, and platform may lead to CNV detection differences. It is also possible that a long term of selection for disease resistant in the lines of chickens led to many lineage-specific events of CNVs.
CNVs are found in relation with disease resistance in both humans and bovine (Gonzalez et al. 2005; Hou et al. 2011). To evaluate the functions of CNVs in chickens, we examined the CNVs profiles in two highly inbred lines (L63 and L72) and two recombinant congenic strains (RCS-L and RCS-M) derived from them in this study. The rational was that because the susceptibility to MD was known ranking as L72 > RCS-M > RCS-L > L63, the genomic structure variations between RCS-M and RCS-L inherited from the L63 and L72 may account for, in part, the different susceptibility to MD. In our experiment, we indeed detected the CNVs of L63 and L72 shown in the RCS-L and RCS-M genome. Most of CNVs in RCS-L (91.7% of total CNVs) and RCS-M (66.7% of total CNVs) were transmitted from L63 and L72 as expected, and presumably most of CNVs inherited from the L63 may be contributable to MD resistance in RCS-M and RCS-L as compared to CNVs from the L72. The CNVs that were only indentified in the RCS-L and RCS-M are considered as lineage-specific CNVs, which may also explain the phenotypic variations between the two RCSs.

We further explored the potential functionality of the CNVs that are differentially presented between L63 and L72. These CNVs found encompassed genes involved in immune response, the MHC class I protein complex, and antigen processing and presentation. Because the same MHC haplotype B2 was found in L63 and L72 chickens (Davison and Nair 2004) and there were no DNA sequence variations as L72 the same MHC haplotype B2 was found in L63 and L72 chickens protein complex, and antigen processing and presentation. Because considering benefit of disease resistance in both humans and bovine (Gonzalez et al. 2005; Hou et al. 2011). To evaluate the functions of CNVs in chickens, we examined the CNVs profiles in two highly inbred lines (L63 and L72) and two recombinant congenic strains (RCS-L and RCS-M) derived from them in this study. The rational was that because the susceptibility to MD was known ranking as L72 > RCS-M > RCS-L > L63, the genomic structure variations between RCS-M and RCS-L inherited from the L63 and L72 may account for, in part, the different susceptibility to MD. In our experiment, we indeed detected the CNVs of L63 and L72 shown in the RCS-L and RCS-M genome. Most of CNVs in RCS-L (91.7% of total CNVs) and RCS-M (66.7% of total CNVs) were transmitted from L63 and L72 as expected, and presumably most of CNVs inherited from the L63 may be contributable to MD resistance in RCS-M and RCS-L as compared to CNVs from the L72. The CNVs that were only indentified in the RCS-L and RCS-M are considered as lineage-specific CNVs, which may also explain the phenotypic variations between the two RCSs.

We further explored the potential functionality of the CNVs that are differentially presented between L63 and L72. These CNVs found encompassed genes involved in immune response, the MHC class I protein complex, and antigen processing and presentation. Because the same MHC haplotype B2 was found in L63 and L72 chickens (Davison and Nair 2004) and there were no DNA sequence variations of the MHC B-FIV identified (Vallejo et al. 1998), for a long time the MD resistance in L63 was attributable to non-MHC genomic variation. The CNV identified in this study indicated that MHC may also contribute to the primarily non-MHC–associated resistance but in a CNV manner instead of a single-nucleotide polymorphism or insertion/deletion polymorphism. A similar case also was found in other species, such as rat (Roos and Walter 2005) and Rhesus macaque (Otting et al. 2005). Generally speaking, the high level of MHC polymorphism is considered beneficial for disease resistance (Wallny et al. 2006). In humans, the lower copy number of MHC is associated with the risk of systemic lupus erythematosus susceptibility (Yang et al. 2007). However, in Tasmanian devils, less MHC may help the animals escape from the disease epidemic (Siddle et al. 2010). The results in our case are similar to the Tasmanian devils.

The question now is how the CNVs differentially represented between the resistant and susceptible lines of chickens modulate disease resistance or susceptibility. One aspect is clear, that is, the gain or loss of a DNA region would influence the expression of the genes in which the CNVs are located. Some researchers found a complicated relationship between gain or loss of CNVs and gene expression in human (Beckmann et al. 2007; Henrichsen et al. 2009; Stranger et al. 2007). In this study, we found that CNVs were positively correlated with gene expression, indicating that the CNVs may influence the host in MD resistance or susceptibility through regulating the expression of the genes.

In the meantime, to confirm the power of disease resistance breeding that may also be shown in other genomic variants, we overlapped the CNVs with the MD-related QTL regions that were identified in previous study. Of all the candidate MD-resistant and -susceptible CNVs, 25% (7/28) of them are overlapped with the QTL. Five functional gene were found located in these overlapped regions, namely LIMS1, MYLK4, C3orf77, FZD6, and BAALC. Three of them were reportedly being related with cancers. LIMS1 was found up-regulated in cancer samples (Wang-Rodriguez et al. 2002) and is required for the apoptosis resistance of cancer cells through involving with the ERK-Bim pathway (Chen et al. 2008). BAALC was found overexpressed in a subset of acute leukemias (Baldus et al. 2003; Tanner et al. 2001). However, the FZD6 functions as an inhibitor of cancer cell transformation by negatively regulating canonical Wnt/β-catenin signaling pathway (Golan et al. 2004).

In conclusion, by using aCGH, we identified a total of 28 candidate CNVs, which may contribute to MD resistance and/or susceptibility in chickens, within which approximately 25% were overlapped with previous identified MD-related QTL. A positive correlation between CNVs and expression of genes encompassing the CNVs was observed, which suggested that the candidate CNVs may influence the susceptibility to MD through regulation of the gene expression. These findings provided additional information elucidating possible mechanisms underlying genetic resistance to MD, which may help to improve strategies to better control of MD in poultry.

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and wrote the paper. Y.Y. extracted DNA. S.C. and H.M.Z. collected samples and revised the paper. N.Y. and H.M.Z. revised the paper. J.Z.S. designed the experiments and revised the paper.

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